

GENETIC ANALYSIS, INHERITANCE AND STABILITY OF MUTATION-BASED  
HERBICIDE TOLERANCE IN COTTON (*GOSSYPIMUM HIRSUTUM* L.)

A Dissertation

by

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## ABSTRACT

The evolution of herbicide-resistant weed species in cotton production has created a need for new herbicide technology tools. Herbicide technologies not classified as genetically modified by recombinant DNA can provide tools with less associated registration and development costs and regulatory and market barriers. Research herein aims to advance herbicide crop tolerance through improvement and genetic analysis of mutation derived herbicide tolerance in cotton. Germplasm exhibiting elevated tolerance to the imidazolinone class of herbicides has been previously identified after mutagenesis with ethyl methanesulfonate (EMS). However, the physiological basis, genetic behavior, and potential for herbicide tolerance improvement are not fully understood and studies were designed to elucidate these factors.

Three lines (EM<sub>4</sub>-3-1-1, EM<sub>4</sub>-3-1-2, and SCM<sub>3</sub>-4-3-1) show high levels of imazamox tolerance. Data indicate that yield for all EMS treated lines was equal to or greater than their respective non-EMS treated cultivar. EMS treatment had no adverse effects on other cotton fiber properties. In 2012, levels of imazamox herbicide injury were seen at 14 days after application (DAA) ranging from 25-34 per cent. A greater level of injury was observed in 2013 ranging from 30-37% 7 DAA, and from 60-68% 14 DAA. Injury was transient throughout both growing seasons. Acetolactate synthase (ALS) gene sequencing characterized a mutation at Ala122 that is classified as conferring tolerance to imidazolinone herbicides, but was inconsistent in lines evaluated. Sequencing also revealed lines that have a truncated form of the protein in this region that may inhibit

imidazolinone binding to the ALS protein. Chi-square analysis indicated this trait behaves in a simple, dominant fashion. Data from parent-offspring regression analysis indicated moderate correlation between parents and F<sub>2</sub> progeny (53%). Correlation is relatively high between F<sub>2</sub> and F<sub>3</sub> progeny (84%) and demonstrates a strong relationship between these generations. Gain from selection indicates a 13.6% improvement in herbicide tolerance, lending to low progress from selection. These studies have shown that non-transgenic breeding methods can confer and improve imidazolinone herbicide tolerance in cotton, though levels of imidazolinone herbicide injury remained commercially unacceptable.

## DEDICATION

To those who gave me support from afar.

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## NOMENCLATURE

GM	Genetically modified
EMS	Ethyl methanesulfonate
IMI	Imidazolinone
HTC	Herbicide tolerant crops
DNA	Deoxyribonucleic acid
GMO	Genetically modified organism
ALS	Acetolactate synthase
LSU	Large sub-unit
PBO	Piperonyl butoxide
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
RCBD	Randomized complete block design
COC	Crop oil concentrate
DAA	Days after application
ANOVA	Analysis of variance
RNA	Ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
PCR	Polymerase chain reaction

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## CHAPTER I

### INTRODUCTION

United States upland cotton (*Gossypium hirsutum* L.) production occurs in seventeen states across the southern U.S. (Smith 1999). Planted upland cotton hectares was 5.9 and 5.0 million hectares for 2011 and 2012, respectively, and produced approximately 14.7 and 16.3 million bales for 2011 and 2012, respectively (USDA-NASS 2013). Total contribution of U.S. upland cotton to the U.S. economy is estimated at \$100 billion (National Cotton Council 2013). The state of Texas has contributed about 33% of total production from 2010 to 2012 (USDA-NASS 2013), making this region vital to U.S. upland cotton production. In order for Texas cotton to remain competitive in the world marketplace, several breeding and crop protection issues need to be addressed through research.

Plant protection and herbicide tolerance is a vital component of cotton improvement using modern transgenic techniques. The evolution of herbicide-tolerant weed species in cotton production has created a need for new herbicide technology tools (Culpepper et al. 2006; Norsworthy et al. 2008; Steckel et al. 2008; Buerkle 2011). While new genetically modified (GM) herbicide traits are being developed to combat weed resistance, development costs are high and regulation immense. Commercialization of transgenic traits includes a gauntlet of pre-commercialization regulatory requirements and post-commercialization market restrictions, as well as expenses related to meeting regulatory requirements and product stewardship. Regulatory costs for transgenic

commercialization, estimated at about \$30 million and higher per product, limit the number of commercialized traits to those that have a large monetary payback (Bradford et al. 2005; Devine 2005). Development costs are passed to the producers in the form of technology fees on seed purchases. These hurdles, plus limited access to intellectual property, have been major impediments to creating a more diverse range of herbicide tolerant traits (Clark et al. 2004; Graff et al. 2004). Non-GM herbicide technologies can provide tools that have lower registration and development costs, while also having fewer regulatory and market barriers. The major advantage of mutation based events, notably those derived through treatment with ethyl methanesulfonate (EMS), versus transgenic events is that there are no additional regulatory requirements beyond what is expected from conventionally developed crops (Tan et al. 2005).

Research herein aims to advance non-GM based herbicide crop tolerance through improvement and genetic analysis of mutation derived herbicide tolerance in cotton. Germplasm exhibiting tolerance to the imidazolinone (IMI) class of herbicides has been identified (Bechere et al. 2009). However, the physiological basis, genetic behavior, and potential for herbicide tolerance improvement are not fully understood. Therefore the objectives of these studies were:

1. Determine the level of IMI tolerance among released germplasm lines identified as tolerant to IMI herbicides;
2. Determine the agronomic performance among released EMS-derived IMI tolerant germplasm lines compared with  $M_0$  parents;

3. Determine the mechanism of IMI tolerance by sequencing the ALS gene in released lines to detect possible IMI tolerant mutation event;
4. Develop second and third generation EMS-derived IMI tolerant populations;
5. Determine broad sense heritability and parent-offspring heritability of the EMS-derived IMI tolerance trait;
6. Determine IMI tolerance segregation ratios and patterns using the second and third generation EMS-derived IMI tolerant populations;
7. Determine gain from selection for EMS-derived IMI tolerance;
8. Develop partial on-farm budget analysis for economic justification of novel herbicide technology.

## CHAPTER II

### LITERATURE REVIEW

#### **Cotton Production**

Cotton consists of 50 recognized *Gossypium* species; 45 are diploid ( $2n=2x=26$ ) and 5 are allotetraploid ( $2n=4x=52$ ) (Brubaker et al. 1999; Fryxell 1984; Stewart 1995; Zhang et al. 2005). The majority of cotton fiber production is grown from two allotetraploid species (Stewart 1995). Upland cotton, *G. hirsutum*, is most widely cultivated because of its improved yield potential and environmental adaptability, while *G. barbadense* is grown on a lesser scale for its quality fiber (Zhang et al. 2005). Both *G. hirsutum* and *G. barbadense* originated from tropical climates of Mexico and Peru, respectively. Wild-types have a perennial, indeterminate, and photoperiod sensitive growth habit; however, the plant has been modified over a long period of time to be photoperiod insensitive to maximize production in higher, more temperate latitudes of 45°N to 30°S (Acquaah 2007).

Cotton production can be sensitive to environmental pressures. Water, temperature, disease, insect, and weed pressure can be limiting factors in cotton production. Abiotic stresses such as drought and temperature extremes can affect flowering and subsequent yields adversely. Large advances in pest control strategies have been made in the last fifty years to protect cotton against a range of biotic stresses. This, combined with other genetic improvements, has resulted in increased yield potential (Wells and Meredith 1984; Bridge 1990; Schwartz and Smith 2008). Genetically modified insect and

herbicide tolerance traits in cotton have revolutionized cotton crop protection.

However, a lack of diversity in GM traits, especially herbicide tolerance, has contributed to increased production challenges including the proliferation of herbicide resistant weeds. There are continuous research efforts in herbicide and technology discovery to develop crop protection products and cotton cultivars that meet these changes in production conditions.

### **History of Herbicide Tolerance in Cotton**

The advent of GM herbicide tolerant crops (HTC) is relatively new to seed production and sales, beginning with the introduction of bromoxynil-tolerant cotton in 1995 (Stalker et al. 1996). Although non-GM HTC were available prior to this, few were successful in capturing a large market share. Similarly, HTC that conferred tolerance to selective herbicide chemistries such as bromoxynil also did not capture a large market share, and were only useful where weed spectrums susceptible to those chemistries existed. Not until HTC that conferred tolerance to non-selective herbicides, such as glyphosate and glufosinate, did HTC start to capture above 75% market share in major crop species such as cotton, soybean (*Glycine max*, L.), and canola (*Brassica napus* L.) (Duke 2005).

Herbicide tolerant crops have allowed farmers to alter their production practices in several ways. One of the most beneficial advantages has been the greater promotion of minimum- or no-tillage practices. The use of broad spectrum herbicides greatly reduced the need for pre-plant deep tillage treatments as well as limiting mechanical cultivation



throughout the growing season, which can be destructive to the integrity of soil structure (Dill 2005). This gave producers a positive view of HTC because it allowed them to be better stewards of their long term land investments as well as reducing fuel and equipment usage related to pulling heavy tillage equipment across fields. It has allowed them to simplify production strategies by reducing the number of herbicides they have to apply throughout the season.

Not all consequences of HTC on production practices have been positive. Because glyphosate and glufosinate are not low-use rate herbicides, combined with the continued need in cotton to use pre-emergence herbicides, the volumetric amount of active herbicide product applied has not been reduced due to HTC (Young 2006). There has also been a sharp rise in herbicide resistant weed populations that has rendered HTC technology ineffective in much of the U.S. Cottonbelt. The severity and rapidity of the development of this problem was largely unforeseen 10 years ago (Dill 2005).

### **Breeding for Herbicide Tolerant Crops**

The most common method used to develop HTC has been through genetic modification with recombinant deoxyribonucleic acid (DNA) and gene introgression via backcross breeding. Once a desired trait has been identified and the DNA segment isolated for the gene of interest, it is then integrated into the genome of the target species using *agrobacterium*. The process involves using this bacterium as a host for the trait DNA which is inserted into a cell culture of the plant species. The DNA is then transferred from the bacterium to the plant cells, and the plant species is regenerated

using tissue culture techniques. Since only a few genotypes of cotton have been identified as acceptable candidates for tissue culture, a less desired phenotype is often used for regeneration through embryogenesis to transform the herbicide trait. Once a successful transformation event has been identified in a donor parent plant, backcross breeding is most commonly used to introgress the transgenic trait into elite germplasm. The resulting cultivars are classified as genetically modified organisms (GMO).

Advances also have been made in developing crops tolerant to herbicides that are not considered to be GMO because the development process does not include introgression of DNA from a foreign species. Mutation breeding has proven to be a successful way to achieve non-GM herbicide tolerant traits. This is accomplished usually by chemical mutagens, most notably EMS. The primary objective of artificial mutation induction is to create a plethora of genetic variability in hopes that subsequent selection will allow the plant breeder to find an individual plant tolerant to a particular herbicide. Seeds are exposed to this mutagen, which causes many random, non-directed mutations throughout the genome. These seeds are planted, and resulting M<sub>1</sub> plants treated with the desired herbicide as a selection agent. Plants that show acceptable tolerance levels are then selected and advanced to the next generation where the selection process is repeated. This is continued until a homogenous population with acceptable tolerance is achieved. The most known success with this method has been the Clearfield® tolerant crops which include wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and sunflower (*Helianthus annuus* L.) that are tolerant to IMI herbicides (Tan et al. 2005). The most recent wheat cultivars developed have multiple tolerance mutations on two genome groups (Pozniak

et al. 2004).

Several point mutations have been identified on the acetolactate synthase (ALS) large sub-unit (LSU) that confer tolerance to ALS inhibiting herbicides, e.g., Ala122, Pro197, Ala205, Trp574, and Ser653 (Gressel 2002; Preston and Mallory-Smith 2001; Tranel and Wright 2002; White et al. 2003). Ala122, Ala205, and Ser653 confer tolerance to IMI herbicides but not cross-tolerance to other ALS inhibitors, while Pro197 confers tolerance to sulfonylureas but low tolerance to IMIs (Tranel and Wright 2002; White et al. 2003; Bright et al. 1992; Thill 1997; Jander et al. 2003; Yu et al. 2003). Other mechanisms for herbicide tolerance, such as metabolism and reduced uptake or translocation, have not been classified in developing commercial IMI-tolerant crops but may contribute to selection of tolerant plants during development (Tan et al. 2005).

Tolerance levels can continue to improve by practicing classical breeding methods. Breeding techniques have been used to introgress mutation derived herbicide tolerant traits in tobacco (*Nicotiana tabacum* L.) and soybean (Mazur and Falco 1989; Sebastian et al. 1989). In sunflower, naturally occurring mutants tolerant to IMIs were selected in wild-type populations and introgressed into cultivated types (Al-Khatib et al. 1998).

Other alternative breeding methods have been successful in deriving non-GM herbicide tolerance. Metabolic detoxification of certain herbicides by specific crop species has been the basis of selectivity for commercial herbicides (Mazur and Falco 1989). In wheat, the compound chlorsulfuron is rendered non-toxic through the cytochrome P450 metabolic pathway (Sweetser et al. 1982). Soybean is known to be naturally tolerant to IMI herbicides through its ability to rapidly metabolize and detoxify

the compound (Tecle et al. 1993). However, in most crop species there is not enough genetic variability for naturally occurring metabolic herbicide tolerance to make this an option.

### **Cytochrome P450 Pathway**

Cytochrome P450 monooxygenases (p-450s) belong to the most important phase I enzymatic system involved in herbicide metabolism by weeds and crops (Siminsky 2006). Tolerance to ALS inhibiting herbicides through metabolic detoxification has been linked to the p-450s metabolic pathway in some species (Romesser and O'keefe 1986; O'keefe et al. 1988; Powles and Yu 2010). Cytochrome p-450s in higher plants are contained in some 20 known gene families, resulting in a large number of diverse enzyme chemistries in plants. Xenobiotic detoxification systems in plants largely involve p-450s. In monocot species, more than 12 distinct herbicide metabolism reactions are shown to be mediated by p-450s (Durst and O'Keefe 1995). Studies in cotton have indicated that the use of phorate insecticides, shown to inhibit p-450s metabolism, can act as a safener to the herbicide clomazone by inhibition of toxic clomazone metabolism through this pathway (Ferhatoglu et al. 2005). Animals catalyze the same types of p-450s reactions as plants and some insects metabolize insecticides in this fashion. In agriculture, piperonyl butoxide (PBO) is used as an insecticide synergist to inhibit p-450s mediated detoxification of pyrethroid insecticides (Durst and O'keefe 1995).

Cytochrome p-450s genes are known to be involved in IMI metabolism (Manabe et al. 2007). Cytochrome p-450s have been shown to be involved in herbicide detoxification using *in vivo* approaches for several species (Werch-Reichhart et al. 2000). Increased ALS herbicide injury caused by PBO addition has been reported for barnyardgrass (*Echinochloa phyllopogon* P. Beauv.) (Fischer et al. 2000), canarygrass (*Phalaris minor* Retz.) (Singh et al., 1998), corn (*Zea mays* L.) and soybean (Kwon et al. 1995). Studies also indicate that the effects of p-450s inhibitors evaluated at whole plant level correlate well with their biochemical ability to inhibit herbicide metabolism (Preston et al. 1996).

### **Glyphosate-resistant Palmer Amaranth**

Since the adaptation of transgenic HTC technology, nearly all planted hectares of HTC crops in the U.S. contain tolerance to the herbicide glyphosate (James 2012). Such a wide adaptation to a single mode of action, non-selective herbicide has endangered the continued utility of this technology. Some cotton producers in the U.S. have relied solely on glyphosate applications in a monoculture system for weed control since its introduction in 1997 (Culpepper et al. 2006). There are 24 weed species confirmed to have biotypes that are resistant to glyphosate as of 2013 (Heap 2013). Palmer amaranth (*Amaranthus palmeri* S. Wats.) is a weed species that is economically troublesome and threatening cotton production in the southeastern United States. Palmer amaranth was first reported to be resistant to glyphosate in Georgia in 2005 and resistant biotypes have been reported since 2005 across the Cottonbelt, most recently in Texas (Culpepper et al.

2006; Norworthy et al. 2008; Steckel et al. 2008; Buerkle 2011).

Studies from biotypes of glyphosate-resistant Palmer amaranth from the southeastern U.S. have confirmed the mechanism of resistance to be that of a duplicate gene copy coding for the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) (Gaines et al. 2010). This allows the plants to overcome inhibition of EPSPS by glyphosate simply by producing much more of it. This was a naturally occurring genetic variation exploited by heavy selection pressure from over-use and reduced rate applications of glyphosate. The mechanisms of spread of resistant biotypes are being studied. Beyond independent populations arising in the southeast because of selection pressure, pollen flow and seed transfer have been a significant factor. Pollen of Palmer amaranth has been shown to travel over 91 m (Sosnoskie et al. 2009). Heritability of the resistance also appears to differ within individual plants. Chimera, which is defined by plant DNA being unique to different plant parts such as lateral branches or stems, could also help contribute to the rapid spread (unpublished data). Chimeric bio-types having lateral stems that vary in EPSPS gene copy number would produce some branches that are glyphosate susceptible while other branches are resistant and survive to propagate.

In the West Texas cotton production region, the arid climate, unique production practices, and lower fecundity of weed species in comparison to production in the eastern U.S. have delayed the onset of glyphosate-resistant weeds. However, suspected resistance is spreading rapidly in this region (Cline 2013). Different management strategies, including alternate mode of action HTC technologies, are needed to ensure the sustainability of cotton production in West Texas and throughout the southeastern

United States.

### **Benefits of IMI Herbicides**

In the early history of herbicide discovery, chemistries were discovered that particular crop species would tolerate but economically important weed species would succumb to. However, the large expense and low success rate of discovering new herbicide chemistries has rendered this no longer practical (Devine 2005). There have been few new herbicides introduced in recent years, and the use of a limited number of chemistries has caused negative impacts on the herbicide market. Since the patent on glyphosate expired in 2000, numerous other companies created their own generic, less expensive formulations. This in turn caused the primary manufacturer of glyphosate, Monsanto, to reduce its market price for its versions of glyphosate. Since use rates of other chemistries were already in decline, this price reduction of glyphosate caused a devaluation of the entire herbicide market and the complete loss of some older products (Duke 2011). Companies have few incentives in this market environment for new herbicide chemistry discovery which may be needed to combat weed resistance issues.

This creates a need to develop HTC technologies with existing chemistries, and deploy them in new crops and in new management systems. The IMI class of herbicides, crop tolerance of which have been successfully deployed in Clearfield® crop systems in wheat, sunflower, canola, and rice, has promise to be successfully used in cotton production (Tan et al. 2005).

The IMIs are classified as ALS inhibitors. Acetolactate synthase is the first enzyme to catalyze the biochemical synthesis of the branched-chain amino acids (Shanner et al. 1984; Singh 1999). Acetolactate synthase inhibitors are absorbed by the roots and foliage and trans-located throughout the plant, accumulating in the apical meristems and auxillary buds. They control a broad spectrum of grass and broadleaf weeds, and have a flexible application being able to be applied at preplant, preemergence, or postemergence directed in non-tolerant crops (Senseman 2007). Imazamox (Raptor®) is a newer IMI chemistry that is characterized as having a lower use rate, lower soil half-life, and broader grass control than previous formulations.

#### **ALS Gene Family in *G. hirsutum***

The ALS gene family in *G. hirsutum* has been characterized by Grula et al. (1995). Six different ALS genes have been identified, with four organized as tandem pairs. Two single gene clones have confirmed constitutive expression and are considered to be the main housekeeping forms of the gene. Data indicate that tandem pairs could be assigned to both A and D subgenomes, however specific assignments could not be made. The two housekeeping forms are homologous, each derive from either the A or D genomes and are regulated identically, and are expressed in equal levels in mature leaves, green pericarp, dry seeds and embryogenic callus tissue in cotton (Grula et al. 1995). The expression of the other tandem pairs appears to be much more complex and less clear, but at least 2 and possibly all 4 are functional. However there appears to be a difference in expression of the ‘upstream’ and ‘downstream’ genes of the linked pairs. Upstream



genes are tissue specific and appear to be only expressed in anthers. Downstream gene expression is variable and appears to be expressed more in plant parts where housekeeping genes are expressed the least, and vice versa (Grula et al. 1995). The complex nature of the ALS gene family in *G. hirsutum* could cause difficulty in developing cotton cultivars with an acceptable level of IMI tolerance.

### **Previous Work**

Seed stocks of upland cotton have been developed conferring tolerance to the IMI family of herbicides (Bechere et al. 2010). In 1997 and 1998, seeds of three Texas High Plains conventional cotton cultivars ('SC 9023', PI 590933; 'AFD Rocket'; and 'AFD Explorer') were treated with 2.45% EMS. Beginning in 2000, M<sub>3</sub>, M<sub>4</sub>, M<sub>5</sub>, and M<sub>6</sub> generations were screened and selected for tolerance to IMI herbicides. In 2004, M<sub>6</sub> and M<sub>7</sub> lines with an elevated level of IMI tolerance were identified. Selected lines were subjected to five rates of imazamox at 0, 88, 175, 350, and 700 g a.i. ha<sup>-1</sup>. It was reported that these EMS treated lines exhibited elevated levels of imazamox tolerance (Bechere et al. 2009). Four imazamox tolerant cotton genetic stocks, SCM<sub>3</sub>-4-3 (Reg. No. GS-3, PI 657941), SCM<sub>3</sub>-7-3 (Reg. No. GS-4, PI 657942), RM<sub>3</sub>-8-1 (Reg. No. GS-5, PI 657943), and EM<sub>4</sub>-3-1 (Reg. No. GS-6, PI 657944) were jointly released from this material by Texas Tech University Department of Plant and Soil Science, and USDA-ARS Crop Genetics and Production Research Unit, Stoneville, MS, in July of 2009 (Bechere et al. 2010). Preliminary studies by Bechere et al. (2009) indicate that the four released genetic stocks exhibit elevated levels of imazamox tolerance conferred by a

partially dominant, single gene. Data also indicate that possible tolerance genes present were either at the same locus or tightly linked.

## **Heritability**

The heritability of a trait is an estimate of the proportion of the genotypic variation to the phenotypic variation, of which the phenotype is a product of the genotype and environmental interaction (Bernardo 2002; Falconer and Mackay 1996; Fehr 1991; Hallauer and Miranda 1981). This measure helps to understand the contributions of genetic and non-genetic factors contributing to phenotypic variation in a population (Bernardo 2002; Hallauer and Miranda 1981; Holland et al. 2003). Heritability estimates are derived by evaluating phenotypes in target breeding environments and are thus specific to the target environment and evaluated population (Hanson 1963; Holland et al. 2003; Wagoire et al. 1999). Therefore, correct sampling of the population and choice of environment is critical in obtaining accurate heritability estimates (Holland et al. 2003; Nyquist 1991).

Heritability in the broad sense measures the ratio of total genetic contribution to the phenotypic variation observed in a given population. This differs from a narrow sense heritability estimate in that it does not distinguish between additive and dominance gene action. Measuring additive genetic effects is beneficial when selecting for quantitative traits because it indicates the value of incremental accumulation of contributing genes in the heterozygous state, and the increased value of contributing genes in the homozygous state (Falconer and Mackay 1996; Fehr 1991; Hallauer and Miranda 1981). However,

knowing additive effects is not as beneficial when breeding for qualitative traits such as herbicide tolerance. Qualitative traits are controlled by only a few genes and thus selection is clearly discernible. A broad sense heritability estimate derived using pure lines is valuable to plant breeders in this instance because the trait is qualitative and incremental changes within a population are not germane to the trait's expression and because it provides some indication of the impact of the environment on the expression of a qualitative trait determined by genes in the homozygous condition. For traits such as herbicide tolerance that can be heavily influenced by environmental pressure such as soil conditions, water availability, and temperature, the extent of this interaction allows breeders to know to what degree mitigating environmental variances is necessary for accurate phenotyping.

### **Economic Justification**

Glyphosate-resistant Palmer amaranth is one of the most economically damaging weeds in the U.S. (Beckie 2011). With the advent of glyphosate-resistant Palmer amaranth throughout the Cottonbelt (Culpepper et al. 2006; Norworthy et al. 2008; Steckel et al. 2008; Buerkle 2011), producers now face additional costs in crop protection to protect yield potential. In Texas it has been shown that Palmer amaranth plant densities of 1 to 10 plants  $9.1 \text{ m}^{-2}$  can reduce cotton lint yields by 11 to 50%, respectively (Morgan et al. 2001). In addition to competition for resources needed for plant growth Palmer amaranth can also impact yields through allelopathy (Bradow and Connick 1987), and in subsequent seasons as research has indicated that the weed can be

a host to various nematode pests (Davis and Webster 2005).

Glyphosate-resistant Palmer amaranth control requires integration of non-herbicidal controls (Ward et al. 2013), and hand cultivation for removal can cost approximately \$39.53 ha<sup>-1</sup> (Smith and Yates 2013) for a single application and may need to be repeated several times throughout the growing season. However, nonchemical controls have also been shown to be inconsistent (Sosnoskie et al. 2012). To control glyphosate-resistant Palmer amaranth and protect cotton yield potential, adoption of cotton varieties with multiple stacked herbicide tolerant traits is needed along with adaptation of proper resistance management practices (Ward et al. 2013). For GM herbicide technology, producers in the Texas High Plains pay from \$1.53 to \$1.93 ha<sup>-1</sup> in technology fees, depending on plant densities under non-irrigated or irrigated production systems, respectively (Smith and Yates 2013). Because mutation-based HTC technology would reduce producer costs through the absence of technology fees, it could provide a cost effective option for cotton producers on the Texas High Plains.

## CHAPTER III

### PARENTAL SELECTION AND STABILITY

#### **Previous Work**

Seed stocks of upland cotton have been developed conferring tolerance to the IMI family of herbicides (Bechere et al. 2010). In 1997 and 1998, seeds of three Texas High Plains conventional cotton cultivars (SC 9023, AFD Rocket, and AFD Explorer) were treated with 2.45% EMS. Beginning in 2000, M<sub>3</sub>, M<sub>4</sub>, M<sub>5</sub>, and M<sub>6</sub> generations were screened and selected for tolerance to IMI herbicides. In 2004, M<sub>6</sub> and M<sub>7</sub> lines with an elevated level of IMI tolerance were identified. Selected lines were subjected to five rates of imazamox at 0, 88, 175, 350, and 700 g a.i. ha<sup>-1</sup>. It was reported that these EMS treated lines exhibit elevated levels of imazamox tolerance (Bechere et al. 2009). Four imazamox tolerant cotton genetic stocks, SCM<sub>3</sub>-4-3 (Reg. No. GS-3, PI 657941), SCM<sub>3</sub>-7-3 (Reg. No. GS-4, PI 657942), RM<sub>3</sub>-8-1 (Reg. No. GS-5, PI 657943), and EM<sub>4</sub>-3-1 (Reg. No. GS-6, PI 657944) were jointly released from this material by Texas Tech University Department of Plant and Soil Science, and USDA-ARS Crop Genetics and Production Research Unit, Stoneville, MS, in July of 2009 (Bechere et al. 2010). Preliminary studies by Bechere et al. (2009) indicate that the four released genetic stocks exhibit elevated levels of imazamox tolerance conferred by a partially dominant, single gene. Data also indicate that possible tolerance genes present were either at the same locus or tightly linked.

Imidazolinone tolerance of these previously developed lines has not been at sufficient levels to draw commercial interest. It is necessary to ascertain field tolerance levels of parental germplasm as a baseline for further improvement. Therefore, field efficacy trials were initiated that included IMI treated and non-IMI treated parents to evaluate tolerance of selected IMI-tolerant parental germplasm. For commercial acceptance it is also necessary to determine if EMS treatment had any adverse effects on cotton agronomic properties, namely yield and fiber quality parameters. To evaluate this, field equivalency trials were initiated that included EMS treated, selected parental germplasm and the respective non-EMS treated cultivars used to develop parents.

## **Efficacy and Equivalency Field Trials**

### **Parental Selection**

Greenhouse studies were initiated in the summer of 2010 in Lubbock, TX, to screen 31 previously derived IMI-tolerant mutant lines for potential parental selection. These lines had improved yield data from previous field studies (data not shown). Fifty plants of each line were arranged in a Completely Randomized Design and imazamox was applied using a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup> at a rate of 88 g a.i. ha<sup>-1</sup> (2x recommended rate) when plants contained 4 to 6 true leaves. Three lines (Table 1) showed elevated levels of visual imazamox tolerance (>95%) of which six siblings were selected from each line. Selected plants were potted in 6 L pots and grown to maturity under greenhouse conditions (30 C and watered as needed). These plants were maintained for seed production to be used in field trials.

Table 1. Selected parental genotypes from IMI screening selection and respective non-EMS treated cultivar.<sup>a,b</sup>

Selected IMI tolerant lines	Non-EMS treated cultivar
EM <sub>4</sub> -3-1-1	AFD Explorer
EM <sub>4</sub> -3-1-2	AFD Explorer
SCM <sub>3</sub> -4-3-1	Seed Co 9023

<sup>a</sup>Selected plants received imazamox at 88 g a.i. ha<sup>-1</sup>.

<sup>b</sup>Screening conducted in greenhouses in Lubbock, TX in summer 2010.

### Materials and Methods

IMI-tolerant parental lines selected from greenhouse screenings were evaluated in a field stability analysis that included equivalency and efficacy studies. Studies were conducted at the Texas A&M Agrilife Research and Extension Center in Lubbock, TX, where soils are classed as an Olton clay loam. Plots were furrow irrigated and hand weeded. In 2012, the first freeze occurred on October 8<sup>th</sup> and no harvest aid was applied. Plots were harvested using a two-row mechanical plot stripper.

In the efficacy study, parental lines were planted on May 7<sup>th</sup> in 2012 and June 5<sup>th</sup> in 2013 in a randomized complete block design (RCBD) with 4 replications. Plots were 4 rows, 5.8 m x 4 m. Treatments were applied to the center 2 rows with a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup> at when plants contained 4 to 8 true leaves on 21 June and 9 July in 2012 and 2013, respectively. Treatments included imazamox at 176 g a.i. ha<sup>-1</sup> (4x recommended rate) plus crop oil concentrate (COC) at 1% v/v and non-sprayed. Data collection included visual injury ratings taken 7, 14, and 69 days after

application (DAA) in 2012 and 7 and 14 DAA in 2013, plus lint yield and fiber quality parameters in 2012. The center 2 rows of each plot were harvested on 30 November 2012.

In the equivalency study, parental lines and their respective non-EMS treated parents were planted in a RCBD with 4 replications in Lubbock, TX on 7 May, 2012 in 4 row plots 5.8 m long by 2 m wide. No herbicide treatment was applied. Plots were harvested on November 30<sup>th</sup>, 2012. Lint yield and fiber quality parameters were analyzed.

### Statistical Analysis

Injury, yield, and fiber quality parameters were analyzed by analysis of variance (ANOVA) using PROC MIXED and PROC GLM (SAS Institute 2010). Shapiro-Wilk and Kolmogorov-Smirnov tests for normality were conducted prior to statistical analysis. Transformation was performed to ensure normality of data when necessary. Transformed data were back transformed for purpose of presentation. Levene's test for homogeneity of variance was used to determine if data could be combined across years. Treatment means were separated using Fisher's Protected LSD ( $P \leq 0.05$ ).

## **Results and Discussion**

### Equivalency

Yield, lint turnout, fiber strength, length, and micronaire were analyzed to determine if EMS treatment caused adverse effects to these properties of cotton production. Yield data indicate that all EMS treated lines had yield that was equal to or greater than their



respective non-EMS treated cultivar (Table 2). Data also indicate that EMS treated lines were comparable in yield to a standard Texas High Plains cultivar FIBERMAX 958 ‘FM 958’ (PVP200100208). Lower yields in SC 9023 could be due to seed quality issues with older seed stocks being utilized for this study. No differences were indicated between lines evaluated for lint turnout, fiber strength, fiber length, or micronaire properties (data not shown).

Table 2. Equivalency yield in kg ha<sup>-1</sup> at Lubbock, TX in 2012.<sup>a,b</sup>

Line	Yield in kg ha <sup>-1</sup>
FM 958	1100 a
SCM <sub>3</sub> -4-3-1	1000 ab
EM <sub>4</sub> -3-1-2	990 ab
EM <sub>4</sub> -3-1-1	850 ab
AFD Explorer	750 b
Seedco 9023	330 c

<sup>a</sup>No herbicide treatment applied.

<sup>b</sup>Values with the same letter in the same column are not different ( $P \leq 0.05$ ).

### Efficacy

Visual injury ratings were measured from 0% (no injury) to 100% (plant death) 7, 14, and 69 DAA in 2012 (Table 3) and 7 and 14 DAA in 2013 (Table 4). Combined injury analysis indicate year to be significant therefore, data are presented by year. All sprayed treatments had greater injury than non-sprayed controls for all evaluation times in 2012 and 2013 (data not shown). Yield, fiber strength, fiber length, and micronaire properties were analyzed for 2012. Data analysis indicated no differences among lines

or between sprayed and non-sprayed treatments for fiber strength, fiber length, or micronaire properties (data not shown).

Table 3. Herbicide injury and cotton lint yield from efficacy study at Lubbock, TX in 2012.<sup>a,b</sup>

Line	Injury (%)			Yield( kg ha <sup>-1</sup> )
	7DAA <sup>c</sup>	14DAA	69DAA	162DAA
EM <sub>4</sub> -3-1-1	15.0 a	32.5 a	15.0 a	659.1 a
EM <sub>4</sub> -3-1-2	13.8 a	33.8 a	16.3 a	799.2 a
SCM <sub>3</sub> -4-3-1	15.0 a	25.0 a	3.0 b	791.3 a

<sup>a</sup>All treatments received imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>Values with the same letter in the same column are not different (P≤0.05).

<sup>c</sup>DAA: Days after application.

Table 4. Herbicide injury from efficacy study at Lubbock, TX in 2013.<sup>a,b</sup>

Line	Injury(%)	
	7DAA <sup>c</sup>	14DAA
EM <sub>4</sub> -3-1-1	36.7 a	67.5 a
EM <sub>4</sub> -3-1-2	29.2 a	59.2 a
SCM <sub>3</sub> -4-3-1	34.2 a	63.3 a

<sup>a</sup>All treatments received imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>Values with the same letter in the same column are not different (P≤0.05).

<sup>c</sup>DAA: Days after application.

In 2012, injury was transient and plants appeared to recover from injury sustained. Yield was not affected by 162 DAA (Table 3) with the exception of EM<sub>4</sub>-3-1-1, which showed significantly lower yields when sprayed with imazamox (Table 5). However, a trend in yield reduction is evident between sprayed and non-sprayed treatments in all lines. Levels of injury were seen at 14 DAA ranging from 25-34%. Greater levels of

injury were seen in 2013. Injury ranged from 30-37% 7 DAA, and from 60-68% 14 DAA. The increase in injury from 2012 to 2013 is likely due to increased rainfall in 2013 allowing an increase in herbicide uptake and translocation in the plant. Rainfall totals at the Texas A&M Agrilife Research and Extension Center in Lubbock, TX for January through July in 2012 and 2013 were 15 cm and 22 cm, respectively (on farm data). Although data indicate that plants show the ability to recover from injury sustained from imazamox application, level of injury observed and a trend of yield reduction raises questions about acceptable IMI-tolerance being conferred, and would likely not be commercially viable.

Table 5. Cotton lint yield in kg ha<sup>-1</sup> from efficacy study at Lubbock, TX in 2012.<sup>a,b</sup>

	EM <sub>4</sub> -3-1-1	EM <sub>4</sub> -3-1-2	SCM3-4-3-1
Control	878.0 a	861.2 a	962.1 a
Imazamox	659.1 b	799.3 a	791.5 a

<sup>a</sup>Imazamox was applied at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>Values with the same letter in the same column are not different (P≤0.05).

## CHAPTER IV

### ORIGINS OF CONFERRED HERBICIDE TOLERANCE

#### **Introduction**

Mutation breeding is a successful method to achieve non-GM herbicide tolerant traits. This is accomplished usually by chemical mutagens, most notably EMS. The primary objective of artificial mutation induction is to create a plethora of genetic variability in hopes that subsequent selection will allow the plant breeder to find an individual plant tolerant to a particular herbicide. The most known success with this method has been the Clearfield® tolerant crops which include wheat, rice, and sunflower that are tolerant to IMI herbicides (Tan et al. 2005).

Several point mutations have been identified on the ALS-LSU that confer tolerance to ALS inhibiting herbicides including Ala122, Pro197, Ala205, Trp574, and Ser653 (Gressel 2002; Preston and Mallory-Smith 2001; Tranel and Wright 2002; White et al. 2003). Ala122, Ala205, and Ser653 confer tolerance to IMI herbicides but not cross-tolerance to other ALS inhibitors, while Pro197 confers tolerance to sulfonylureas but low tolerance to IMIs (Tranel and Wright 2002; White et al. 2003; Bright et al. 1992; Thill 1997; Jander et al. 2003; Yu et al 2003). Other mechanisms for herbicide tolerance, such as metabolism and reduced uptake or translocation, may contribute to selection of tolerant plants during development (Tan et al. 2005).

The ALS gene family in *G. hirsutum* has been characterized by Grula et al. (1995). Six different ALS genes have been identified, with four organized as tandem pairs. Two

single gene clones had confirmed constitutive expression and are considered to be the main housekeeping forms of the gene. Data indicate that tandem pairs could be assigned to both A and D subgenomes, however, specific assignments could not be made. The two housekeeping forms are homologous, each derive from either the A or D genomes and are regulated identically, and are expressed in equal levels in mature leaves, green pericarp, dry seeds and embryogenic callus tissue in cotton (Grula et al. 1995). The expression of the other tandem pairs appears to be much more complex and less clear, but at least 2 and possibly all 4 are functional. However there appears to be a difference in expression of the ‘upstream’ and ‘downstream’ genes of the linked pairs. Upstream genes are tissue specific and appear to be only expressed in anthers. Downstream gene expression is variable and appears to be expressed more in plant parts where housekeeping genes are expressed the least, and vice versa (Grula et al. 1995).

Other alternative breeding methods have been successful in deriving non-GM herbicide tolerance. Metabolic detoxification of certain herbicides by specific crop species has been the basis of selectivity for commercial herbicides (Mazur and Falco 1989). In wheat, the compound chlorsulfuron is rendered non-toxic through the cytochrome p-450s metabolic pathway (Sweetser et al. 1982). Soybean is known to be naturally tolerant to IMI herbicides through its ability to rapidly metabolize and detoxify the compound (Teclé et al. 1993).

Cotton lines were selected that have been previously developed to show elevated tolerance to IMI herbicides. It is necessary to elucidate the genetic and physiological mechanisms of this tolerance in order to improve and develop commercially acceptable

IMI tolerant cotton germplasm. Therefore the objective of the following study is to detect any possible mutation event on the ALS-LSU that would confer IMI tolerance.

## **ALS Gene Sequencing**

### Materials and Methods

Plant tissue was collected from selected IMI-tolerant lines from previous studies conducted under greenhouse culture in Lubbock, TX, as well as an IMI-susceptible variety. Tissue from four separate specimens of EM<sub>4</sub>-3-1-1, EM<sub>4</sub>-3-1-2, SCM<sub>3</sub>-4-3-1, and FM 958 were sampled and stored in dry ice. Ribonucleic acid (RNA) extraction was performed on samples to derive complementary DNA (cDNA) for polymerase chain reaction (PCR) and cloned plasmid DNA products to be used in ALS-LSU gene sequencing.

Fifty mg of frozen tissue from each sample was placed in a 2 ml centrifuge tube containing cubic zirconium beads with the tubes held on dry ice. Methanol (100%) was added to each tube at a volume of 500  $\mu$ l and ground in a FastPrep®-24 instrument for 60 seconds. Resulting solution was centrifuged and supernatant removed. This process was repeated to insure removal of gossypol content in older cotton tissue samples. Five-hundred  $\mu$ l of RNA extraction buffer solution (100  $\mu$ M LiCl, 100  $\mu$ M Tris-HCl, pH 8.0, 1% SDS, and 10  $\mu$ M EDTA, plus 10 $\mu$ l/ml of proteinase K solution containing 200 ng/ml proteinase K final concentration, plus 10 $\mu$ L/mL 2-mercaptoethanol) was added and homogenized in FastPrep®-24 instrument for 60 seconds. Samples were placed at 65 C for >10 min to allow proteinase K digestion of proteins. Five-hundred  $\mu$ l of

phenol/chloroform/isoamyl alcohol 25:24:1 mixture was added to each tube and vortexed. Tubes were centrifuged for 60 seconds at 5,000 rpm to separate phases. The aqueous layer in each tube was transferred to a new tube containing 500 µl of isopropanol, mixed, and centrifuged at 12,000 rpm for 10 min to derive pelletized nucleic acid; pellets were washed in 70% ethanol. Pellets were re-suspended in 100 µl of RNase-free water at 65 C for 5 to 10 min. RNA content was quantified using a NanoDrop spectrophotometer and gel electrophoresis analysis. Samples were stored overnight at -80 C.

Synthesis of cDNA and cDNA PCR product was performed using Invitrogen™ cDNA synthesis: Reverse Transcription for Real-Time PCR kit and standard protocol from Life Technologies™. Primers to derive cDNA PCR product and plasmid DNA sequencing were designed using OLIGO 7.0 software and synthesized by Integrated DNA Technologies® (Table 6). Cycling conditions of PCR reaction for cDNA product were 2 minute incubation at 94 C, 40 cycles of 10 second denaturation at 94 C, 10 seconds annealing at 48 C, and 2.5 minute extension at 72 C. Samples were stored at 10 C. PCR products were electrophoresed on agarose gel containing ethidium bromide. Bands containing the PCR product were cut from the gel and agarose digested. DNA was precipitated overnight and centrifuged at 14,000 rpm for 10 min. The resulting pellet was washed in 70% ethanol, centrifuged and air dried. Pellets were re-suspended in 10 µl of RNase-free water for Plasmid DNA cloning. TOPO vectors of plasmid DNA for sequencing were derived for each sample using BigDye® Terminator v3.1 Cycle Sequencing Kit and standard protocol from Applied Biosystems®. Samples were sent to

SeqWright DNA Technology Services Lab in Houston, TX for ALS-LSU gene sequencing.

Consensus sequences were aligned using Seqman NGen® software from DNASTAR, Inc.© and allele number determined. Consensus files were translated in SeqBuilder™ and resulting proteins were aligned in MegAlign™.

Table 6. DNA primer sequences used in PCR for cDNA product synthesis and sequencing of plasmid DNA for ALS LSU sequencing in selected *G. hirsutum* lines.

	Primer	Sequence
cDNA Synthesis	GhALSlsesub-For1	TCTTCCCCTCTCGCTCACCAC
	GhALSlsesub-For2	CTCACCACAAGCCTCTCATCG
	GhALSlsesub-For3	ATGGCGGCTGCCACTTCGAAC
	GhALSlsesub-Rev1	CCATTTGATGCTCATTGAGGTC
	GhALSlsesub-Rev2	CAATATTGTGTTCTTCCATC
	GhALSlsesub-Rev3	ACCCTCTGTGATCACATCTTTG
Plasmid DNA Sequencing	GhALSlsesub seq-For1	GAATGCAATTATAAGTACCGGTG
	GhALSlsesub seq-For2	GTTTGAAGTCTAGTGAGGAG
	GhALSlsesub seq-For3	TGTCCTTCCGCGACACGAGC
	GhALSlsesub seq-Rev1	TCCAATAGCAGCAGGCAATC
	GhALSlsesub seq-Rev2	GCATAATTGGCATAACAGTTTC
	GhALSlsesub seq-Rev3	TCAGCGAGACCACTCACCAAG

## Results and Discussion

Consensus ALS-LSU sequence alignment in evaluated lines indicated a single base pair G to A point mutation at nucleotide 337 of reference *G. hirsutum* ALS amino acid sequence (Figure 1). This base pair change results in an amino-acid substitution of Ala122 to Thr122 in lines containing the G to A point mutation (Figure 2). The Ala122-



Thr122 is well documented as conferring tolerance to IMI herbicides, but not other ALS-inhibiting herbicides, in several plant species (Tranel and Wright 2002; White et al. 2003; Bright et al. 1992). This is likely the candidate mutation leading to tolerance of IMI herbicides in selected lines. However, sequence data indicate that the mutation is not consistent within all selected IMI-tolerant lines. This leads to conjecture that selected parental lines were not true breeding for the IMI-tolerant mutation and are still segregating for this trait.

Further information about the nature of the ALS gene in cotton and the IMI-tolerant mutation event can be derived from the partial protein sequence data in Figure 2. As previously described, 6 ALS genes in cotton have been identified with 2 being the main housekeeping forms of the gene (Grula et al. 1995). The allotetraploid nature of *G. hirsutum* can allow one of these genes to be assigned on either the A or D subgenomes. Because these housekeeping forms are constitutive and the most highly expressed, it is likely that these are the genes that were sequenced. At position 108 in Figure 2 protein alignment, a polymorphism can be identified that represents genetic variation due to A and D subgenome copies of the ALS gene. This polymorphism is present in some FM 958 clones and clones of EM<sub>4</sub>-3-1-1, and EM<sub>4</sub>-3-1-2, but not the reference *G. hirsutum* or other clones of EM<sub>4</sub>-3-1-1 and EM<sub>4</sub>-3-1-2, and not in SCM<sub>3</sub>-4-3-1. Genetic variation that likely represents the A and D subgenome copies of the ALS gene can be placed in two groups at position 161. It can also be deduced that two regions (position 122 and 154) show clear genetic variation that was likely induced by treatment with EMS, with position 122 being the likely candidate that confers tolerance to IMI herbicides.

It is possible that variation in other lines could cause tolerance to IMI herbicides. For clones 28, 27, and 26 of EM<sub>4</sub>-3-1-2 and clone 12 of EM<sub>4</sub>-3-1-1, there appears to be a sequence deletion that results in a truncated form of the protein. Although this could be due to PCR error, it is more likely that this represents genetic variation resulting from gene copy number because variation also seen at positions 161 and 154 puts the lines with truncated proteins into two groups. Any other scenario would call for the deletion to have occurred simultaneously in both subgenomes and this would be a rare event. The structure of the ALS-LSU protein folds in a way that places the Ala122-Thr122 substitution in a pocket of a proposed key herbicide-binding site (Duggleby et al. 2003), thus, this truncated region could inhibit binding of the IMI herbicide and therefore confer a higher level of IMI-tolerance in these parental lines compared to those without the truncated protein form or the Ala122 point mutation. Further ALS gene sequencing is needed to confirm polymorphisms and limit the possibility of PCR error.

Figure 1. Consensus ALS-LSU sequence alignment showing base pair change that results in amino acid substitution.

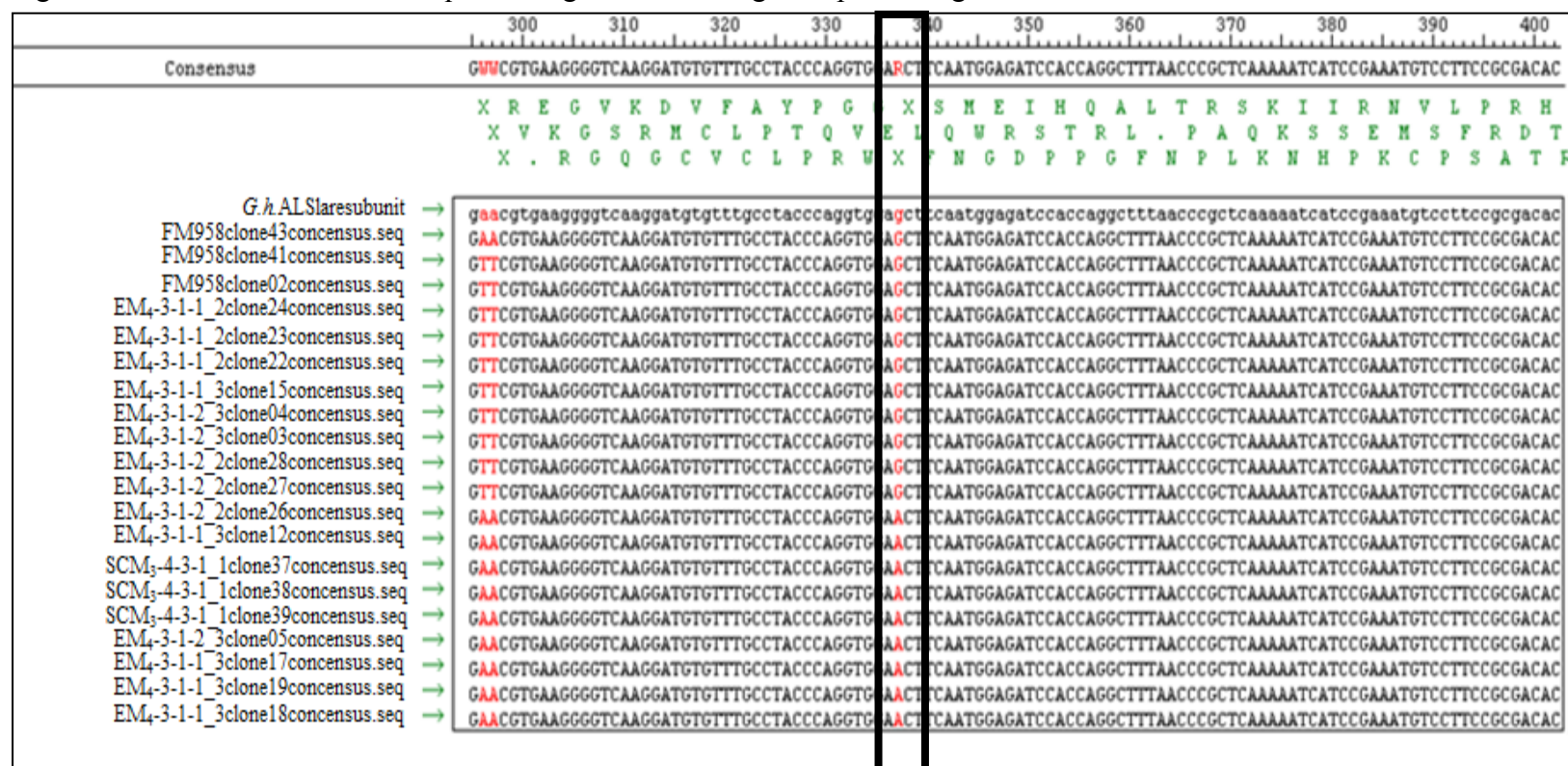
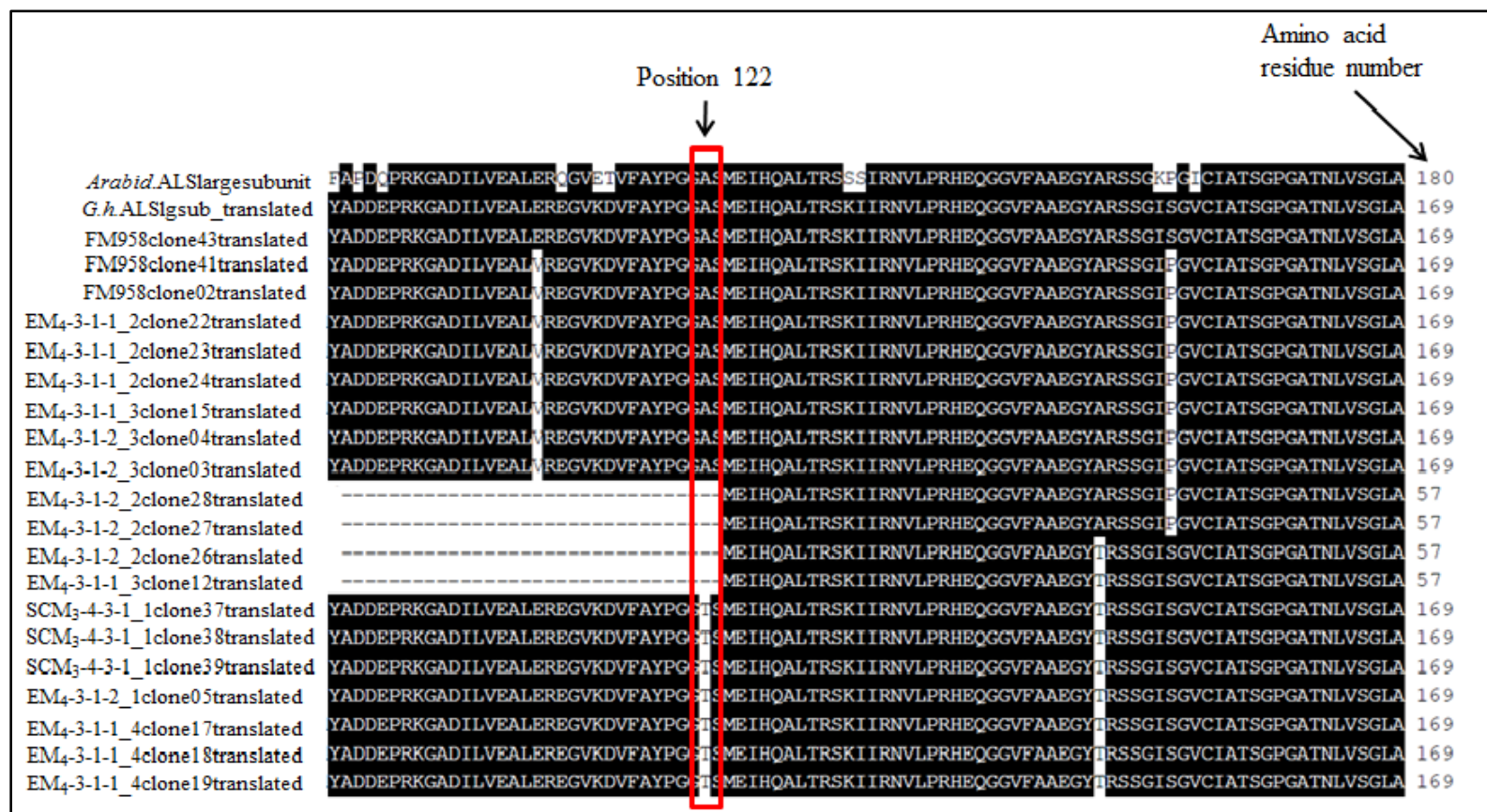


Figure 2. Partial protein alignment from translated consensus sequences showing Ala to Thr mutation at position 122 for clones of three lines.



## CHAPTER V

### HERITABILITY AND IMPROVEMENT

#### **Introduction**

In order to elucidate the genetic behavior of the IMI-tolerant mutation event through generations, studies were designed and initiated to measure heritability in the broad sense through variance analysis and through parent-offspring regression analysis. In order to measure level of improved IMI-tolerance from selection, populations were created to measure gain from selection. Segregation analysis was performed to further elucidate zygoty and IMI-tolerance gene function in parents and subsequent populations.

Heritability in the broad sense ( $H^2$ ) measures the ratio of total genetic contribution to the phenotypic variation observed in a given population and generation. This differs from a narrow sense heritability ( $h^2$ ) estimate in that it does not distinguish between additive and dominance gene action. Measuring additive genetic effects is beneficial when selecting for quantitative traits because it indicates the value of incremental accumulation of contributing genes in the heterozygous state, and the increased value of contributing genes in the homozygous state (Falconer and Mackay 1996; Fehr 1991; Hallauer and Miranda 1981). However, knowing additive effects is not as beneficial when breeding for qualitative traits such as herbicide tolerance. Qualitative traits are controlled by only a few genes and thus selection is clearly discernible. A broad sense heritability estimate derived using pure lines is as valuable to plant breeders in this

instance because the trait is qualitative and incremental changes within a population are not germane to trait expression and because it provides some indication of the impact of the environment on the expression of a qualitative trait determined by genes in the homozygous condition. Variance components are used to derive an estimate of the degree of genetic effects on the phenotype within a generation population. The regression of offspring to parents measures the degree of resemblance between relatives (Bernardo 2002; Fehr 1991; Nyquist 1991). To estimate heritability through this method, data include mean values from parents as well as offspring. A simple linear regression is performed and heritability ( $h^2$ ) is derived from the linear regression coefficient between generational mean values. To understand gene action over several generations, the chi-square test was used to determine plausible allelic ratios. To quantify improvement in a selected population, gain from selection was calculated to measure percent improvement of tolerance from an  $F_2$  population to a selected population of  $F_3$  progeny rows.

### **Population Development**

Greenhouse studies were initiated in the summer of 2010 in Lubbock, TX, to screen 31 previously derived IMI-tolerant mutant lines for potential parental selection. Previous data (not shown) indicated that these lines had improved yield performance under field conditions. Fifty plants of each line were arranged in a completely randomized design and imazamox was applied using a  $CO_2$ -pressurized backpack sprayer delivering  $140\text{ L ha}^{-1}$  at a rate of  $88\text{ g a.i. ha}^{-1}$  (2x recommended label rate) when

plants contained 4 to 6 true leaves. Three lines (Table 1) showed elevated levels of visual imazamox tolerance (>95%) of which six siblings were selected of each line. Selected plants, along with a Texas High Plains standard cultivar FM 958, were potted in 6 L pots and grown to maturity under greenhouse conditions (30 C and watered as needed). Selected IMI tolerant plants (male) were crossed with a respective FM 958 plant (female) to derive F<sub>1</sub> progeny (Figure 3). F<sub>1</sub> progeny seed from each cross combination (2 plants per cross combination) were planted in 6 L pots and grown to maturity under greenhouse conditions to derive F<sub>2</sub> seed. F<sub>1</sub> and F<sub>2</sub> progeny seed were increased in a winter nursery in Tecomán, Mexico to derive F<sub>2</sub> and F<sub>3</sub> generations, respectively, for broad sense and parent-offspring heritability field studies. Increases were controlled self-pollinations, with 25 seed planted in 5 hills for each parental combination. Seeds within each generation were bulked by family. Parental seeds used in these field studies were harvested from selected plants maintained in the greenhouse. For the improvement study, F<sub>2</sub> seeds derived from greenhouse increases of F<sub>1</sub> progeny were bulked by family and planted in a field nursery in Lubbock, TX in 2012. Selected F<sub>3</sub> progeny were derived from individual plant selection in the F<sub>2</sub> nursery and planted in progeny rows in a field nursery in Lubbock, TX in 2013 (Figure 4).

Figure 3. Non-selected population development in greenhouse and winter nursery in Tecomán, Mexico of parents, F<sub>2</sub>, and F<sub>3</sub> seeds used in field heritability trials in Lubbock, TX.

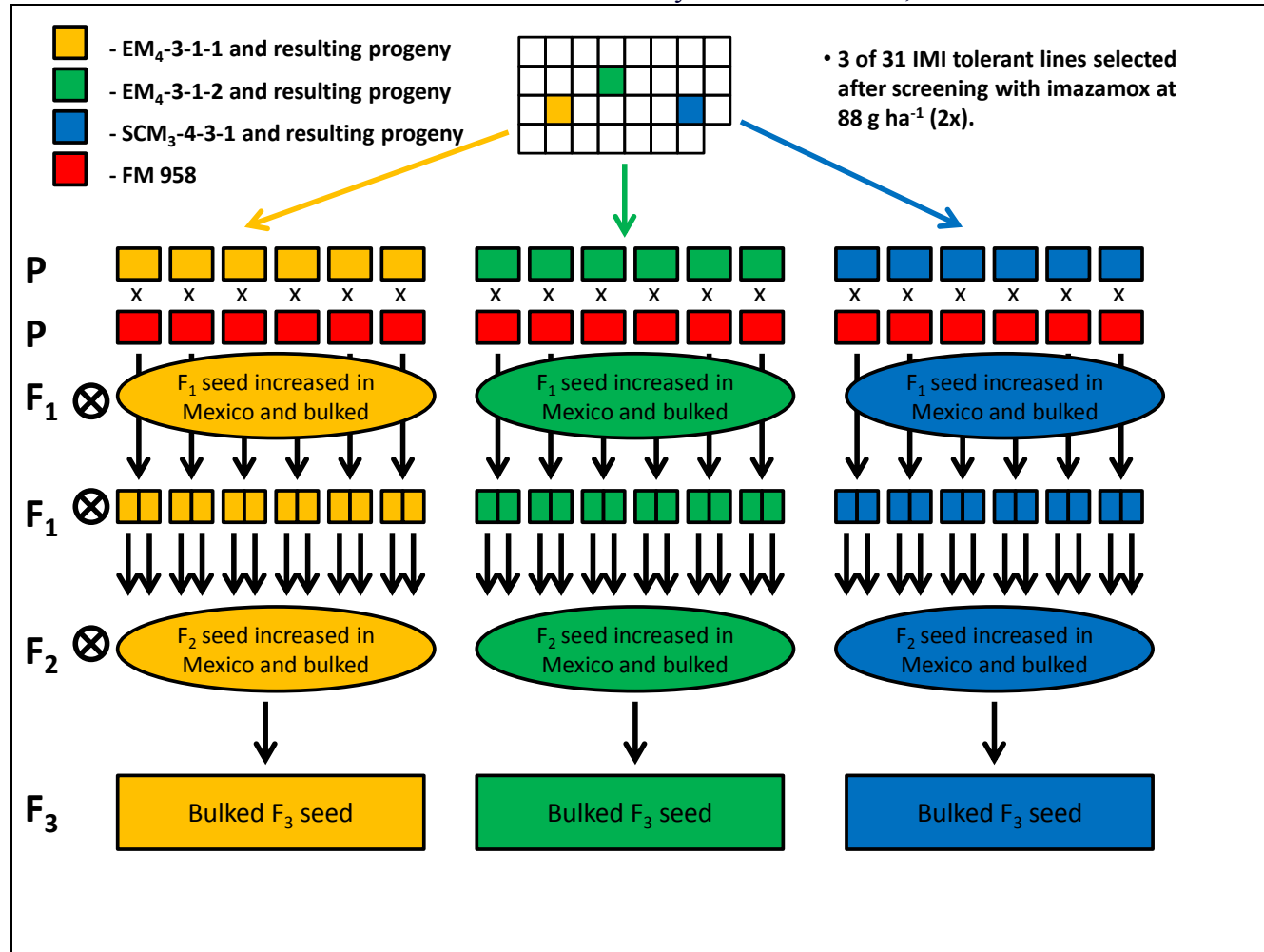
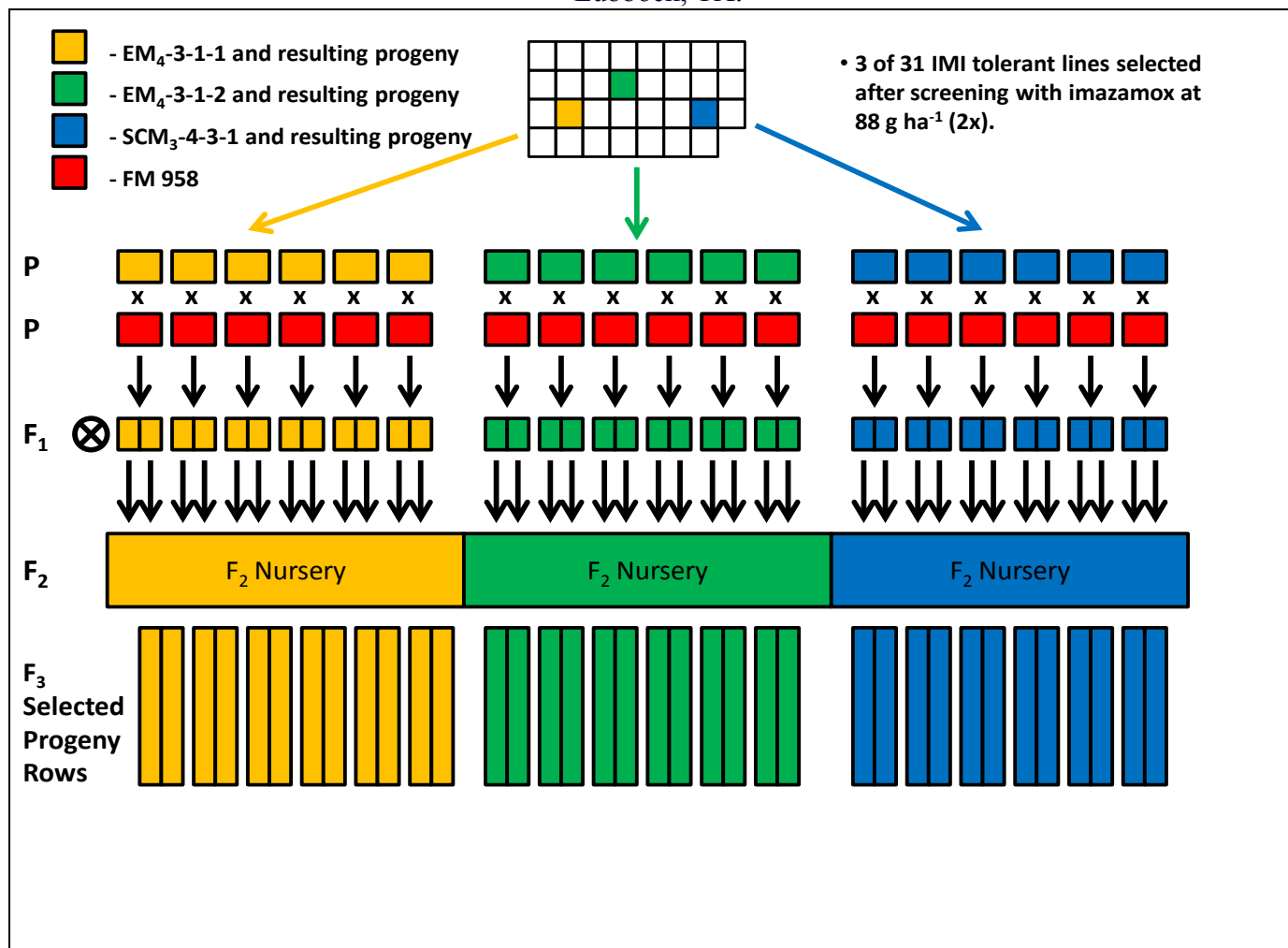




Figure 4. Selected population development of parents, F<sub>2</sub>, and selected F<sub>3</sub> progeny rows in greenhouses and field nurseries in Lubbock, TX.



## **Heritability by Variance Components**

### Materials and Methods

Broad sense heritability ( $H^2$ ) estimates were obtained by the components of variance method using estimates from parent,  $F_2$ , and  $F_3$  generations. No inbreeding was assumed because families were bulked and no family structure was present. Parents (EM<sub>4</sub>-3-1-1; EM<sub>4</sub>-3-1-2; SCM<sub>3</sub>-4-3-1),  $F_2$ , and  $F_3$  entries from each generation family (FM 958 x EM<sub>4</sub>-3-1-1; FM 958 x EM<sub>4</sub>-3-1-2; FM 958 x SCM<sub>3</sub>-4-3-1) were planted in a RCBD with four replications in 2012, and eight replications in 2013 in Lubbock and Lamesa, TX on 7 May and 24 May in 2012, respectively, and 21 May and 8 May in 2013, respectively. Plots were one row, 5.8 m x 1 m. Treatments included imazamox applied at 176 g a.i. ha<sup>-1</sup> (4x recommended rate) plus COC at 1% v/v applied with a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup>. In 2012, treatments were applied when plants contained 4 to 8 true leaves on 21 June and 3 July at Lubbock and Lamesa, respectively. In 2013, treatments were applied when plants contained 8 to 12 true leaves on 8 July and 5 July at Lubbock and Lamesa, respectively. In 2012, data collection included whole plot visual injury ratings at 7, 14, and 62 DAA. In 2013, data collection included whole plot visual injury ratings at 7 and 14 DAA, as well as individual plant ratings at 28 DAA. Visual injury ratings were measured from 0% (no injury) to 100% (plant death). In 2013 herbicide injury was pronounced and discrete and therefore allowed for individual plant ratings of tolerant, intermediate, or susceptible at 28 DAA. The individual plant ratings in 2013 were analyzed separately.

## Statistical Analysis

Injury data were analyzed and variance components derived for each generation population using PROC MIXED (SAS Institute 2010). Broad sense heritability was estimated by entry mean basis over years and location using the equation:

$$H^2 = \frac{Vg}{Vg + \frac{Vgl}{l} + \frac{Vgy}{y} + \frac{Vgyl}{ly} + \frac{Ve}{lyr}}$$

Where Vg = variation due to genotype

Ve = variation due to environment

Vgl = variation due to genotype by location interaction

Vgy = variation due to genotype by year interaction

Vgyl = variation due to genotype by year by location interaction

l = number of locations

y = number of years

r = replications

In 2013,  $H^2$  was estimated by individual plant ratings within entry. This analysis did not contain year as a covariate.

## Results and Discussion

For the purposes of presenting  $H^2$  of IMI herbicide injury, estimates from the 14 DAA ratings are presented (Table 7). This choice in timing for injury observations is the result of results from field efficacy studies that revealed injury to be most pronounced during this time, and therefore is the most impactful measurement to present. Broad sense heritability estimates of zero in all generations indicate that there was no genetic variance present between lines in each respective generation. This results from the Vg variance component having a value of zero in all generational analysis (Table 8).

However, analysis of fixed effects did reveal significance for location, year and year x

location for parent and  $F_3$  generations, and location and year x location for the  $F_2$  generation (Table 9). This reveals that a large genotype x environment interaction was occurring in all generations, which has the ability to mask any potential genetic effects, thus producing an estimate of zero for  $V_g$  and subsequently a value of zero for  $H^2$ . In addition, a large proportion of variance is attributed to residual error (Table 8) and could also work to mask potential genetic effects. Herbicide uptake and translocation is dependent on the growth activity of the plant which is determined largely, especially in the Texas High Plains, by water availability. Rain totals from January through July for Lubbock were 15 and 22 cm in 2012 and 2013, respectively, and 15 and 18 cm for Lamesa in 2012 and 2013, respectively (on farm data). Although plots were irrigated as needed, irrigation is a rainfall supplement and not a rainfall replacement. Thus these precipitation differences between year and location could have a large effect on herbicide uptake and injury expression. In addition, these totals are below the yearly average rainfall for this time period at both locations (26 and 27 cm at Lamesa and Lubbock, respectively).

Table 7. Broad sense heritability ( $H^2$ ) estimates for lines and progeny of EM<sub>4</sub>-3-1-1, EM<sub>4</sub>-3-1-2, SCM<sub>3</sub>-4-3-1 from mean injury at 14 DAA at Lubbock and Lamesa, TX, in 2012 and 2013.<sup>a,b</sup>

	$H^2$		
	Parent	$F_2$	$F_3$
Injury 14DAA	0%	0%	0%

<sup>a</sup>Treatments included imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>DAA: Days after application.

Table 8. Covariance parameter estimates for plot injury 14 DAA at Lubbock and Lamesa, TX, in 2012 and 2013.<sup>a</sup>

	Parent	F <sub>2</sub>	F <sub>3</sub>
Genotype	0	0	0
Location	0	0	0
Location x Genotype	0	1.88	0
Year	0	19.46	32.15
Year x Genotype	151.58	7.23	5.10
Year x Location	72.24	2.95	0
Block(Year x Location)	3.10	0	10.51
Year x Location x Genotype	22.09	0	15.45
Residual	149.60	69.79	98.22

<sup>a</sup>DAA: Days after application.

Table 9. Fixed effects p-values for plot injury 14 DAA at Lubbock and Lamesa, TX, in 2012 and 2013.<sup>a</sup>

	Parent	F <sub>2</sub>	F <sub>3</sub>
	N = 107	N = 108	N = 108
Genotype	0.22	0.71	0.88
Location	<.0001 **	0.03 *	0.03 *
Location x Genotype	0.11	0.78	0.40
Year	<.0001 **	0.16	0.03 *
Year x Genotype	0.31	0.54	0.85
Year x Location	<.0001 **	0.01 **	0.01 **
Year x Location x Genotype	0.14	0.63	0.19

<sup>a</sup>DAA: Days after application.

\*Significant at the 0.05 level of probability.

\*\*Significant at the 0.01 level of probability.

In an effort to minimize environmental variance and to have more power to detect statistical significance,  $H^2$  was also calculated using individual plant tolerant ratings of susceptible, intermediate, and tolerant at Lubbock and Lamesa for 2013 by removing year as a variance component. In the parents,  $F_2$ , and  $F_3$  generations, 1473, 1421, and 1502 plants were rated, respectively, at combined locations. These ratings were not possible to collect in 2012 because injury was not discretely expressed among plants, most likely due to a lack of moisture for plant growth and herbicide uptake and translocation. However, estimates of zero for  $H^2$  were still estimated in each generation due to a value of zero for  $V_g$  (Table 10). High residual error in covariance parameter estimates (Table 11) is present. This could be due to year not being included as a covariance parameter, as well as rating error due to the more discrete rating system of susceptible, intermediate, or tolerant individual plant ratings. The high error present in this model has the potential to mask genetic variance and result in a value of zero for  $V_g$  (Table 11).

Table 10. Broad sense heritability ( $H^2$ ) estimates for lines and progeny of EM<sub>4</sub>-3-1-1, EM<sub>4</sub>-3-1-2, SCM<sub>3</sub>-4-3-1 from individual plant tolerance ratings at 28 DAA at Lubbock and Lamesa, TX, in 2013.<sup>a,b</sup>

	$H^2$		
	Parent	$F_2$	$F_3$
Tolerance 28DAA	0%	0%	0%

<sup>a</sup>Treatments included imazamox at 176 g a.i. ha<sup>-1</sup> plus COC at 1% v/v.

<sup>b</sup>DAA: Days after application.

Table 11. Covariance parameter estimates for individual plant tolerance ratings 28 DAA at Lubbock and Lamesa, TX, in 2013.<sup>a</sup>

	Parent	F <sub>2</sub>	F <sub>3</sub>
Genotype	0	0	0
Location	0.09	0	0
Location x Genotype	0.01	0.01	0.05
Block(Location)	< 0.01	< 0.01	0
Residual	0.95	0.75	0.90

<sup>a</sup>DAA: Days after application.

Table 12. Fixed effects p-values for individual plant tolerance ratings 28 DAA at Lubbock and Lamesa, TX, in 2013.<sup>a</sup>

	Parent		F <sub>2</sub>		F <sub>3</sub>	
	N = 1473		N = 1421		N = 1502	
Genotype	0.0001	**	0.02	*	<.0001	**
Location	<.0001	**	0.65		0.49	
Location x Genotype	0.0009	**	0.001	**	<.0001	**

<sup>a</sup>DAA: Days after application.

\*Significant at the 0.05 level of probability.

\*\*Significant at the 0.01 level of probability.

Quisenberry et al. (1980) reported similar heritability estimates when testing for cotton lint yield at locations in the Texas High Plains region. Data from this study showed zero heritability estimates, with significant year, location, and year by location effects. It was concluded that this large genotype x environment interaction was caused by large differences and deficiencies in precipitation and heat units between years and locations. These results are common throughout the literature, but this is one example of the authors concluding that breeders must be mindful of testing and selecting in regions with large climatic variations, and work to minimize environmental variation to reduce error and increase power to detect true genetic differences. Herbicide activity is greatly dependent on environmental variables, most importantly moisture availability. In Lubbock, rainfall deficits from January to July in 2012 and 2013 were 11 and 9 cm, respectively, from the yearly average for this period. Likewise in Lamesa, rainfall deficits from January to July in 2012 and 2013 were 13 and 5 cm, respectively. Although moisture availability improved in 2013, these deficits and variability inhibit the ability to effectively evaluate herbicide activity and tolerance.

## **Heritability by Parent-Offspring Regression**

### Materials and Methods

Heritability ( $h^2$ ) was estimated by parent-offspring regression analysis to determine the degree of resemblance from parents to progeny. Parents (EM<sub>4</sub>-3-1-1; EM<sub>4</sub>-3-1-2; SCM<sub>3</sub>-4-3-1), F<sub>2</sub>, and F<sub>3</sub> entries from each generation family (FM 958 x EM<sub>4</sub>-3-1-1; FM 958 x EM<sub>4</sub>-3-1-2; FM 958 x SCM<sub>3</sub>-4-3-1) were planted in a RCBD with 4 replications in



2013 in Lubbock and Lamesa, TX, on 21 May and 8 May, respectively. Plots were one row, 5.8 m x 1 m. Treatments included imazamox applied at 176 g a.i. ha<sup>-1</sup> (4x recommended label rate) plus COC at 1% v/v applied with a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup>. Treatments were applied when plants contained 8 to 12 true leaves on 8 July and 5 July, 2013 at Lubbock and Lamesa, respectively. In 2013 herbicide injury was pronounced and discrete and allowed for individual plant ratings of tolerant, intermediate, or susceptible at 28 DAA, and was used for this regression analysis.

### Statistical Analysis

Regression values were calculated by regressing F<sub>3</sub> individual progeny tolerance ratings on F<sub>2</sub> individual parent tolerance ratings, and F<sub>2</sub> individual progeny ratings on parental line individual tolerance ratings. Regression was performed using PROC REG (SAS Institute 2010). Shapiro-Wilk and Kolmogorov-Smirnov tests for normality were conducted.

### Results and Discussion

Plant injury was discrete between plants in 2013, and differences between susceptible, intermediate, and tolerant plants were easily discernible within generation and within plot. For all 3 families, 1473, 1421, and 1502 plants were rated for parents, F<sub>2</sub>, and F<sub>3</sub> generations, respectively, which gives strong statistical power to estimate h<sup>2</sup>. Data indicate a moderate correlation between parents and F<sub>2</sub> progeny (53%). These

results could be linked to the sequence data in Chapter IV that revealed parental lines selected for these studies were inconsistent for the IMI-tolerant mutation event, and therefore this trait was inconsistently transmitted to the F<sub>2</sub> generation. However, correlation was strong between F<sub>2</sub> plants and F<sub>3</sub> progeny (84%). This demonstrates a strong relationship between generations and resemblance of plant tolerance appears to be transmitted from F<sub>2</sub> plants to F<sub>3</sub> progeny. This lends to the IMI-tolerant mutation behaving in a dominant fashion as it becomes fixed in homozygote state in later generations. Estimating heritability by parent-offspring regression is similar to narrow sense heritability estimates where the degree to which genetic effects are transmitted from one generation to the next is determined.

## **Segregation Analysis**

### Materials and Methods

Greenhouse studies were initiated in the summer of 2010 in Lubbock, TX, to screen 31 previously derived IMI-tolerant mutant lines for potential parental selection. These lines had improved yield data from previous field studies. Fifty plants of each line were arranged in a completely randomized design and imazamox was applied using a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup> at a rate of 88 g a.i. ha<sup>-1</sup> (2x recommended label rate) when plants contained 4 to 6 true leaves. Three lines (Table 1) showed elevated levels of visual imazamox tolerance (>95%) of which six siblings were selected from each line. Selected plants, along with a Texas High Plains standard cultivar FM 958 were potted in 6 L pots and grown to maturity under greenhouse

conditions (30 C and watered as needed). Selected IMI tolerant plants (male) were crossed with a respective FM 958 plant (female) to derive F<sub>1</sub> progeny (Figure 3). F<sub>1</sub> progeny from each cross combination (2 plants per cross combination) were planted in 6 L pots and grown to maturity under greenhouse conditions to derive F<sub>2</sub> plants. F<sub>1</sub> and F<sub>2</sub> progeny were increased in a winter nursery in Tecomán, Mexico to derive F<sub>2</sub> and F<sub>3</sub> generations, respectively. Increases were controlled self-pollinations, with 25 seeds planted in 5 hills for each cross. Seeds within generation were bulked by family. Parents (EM<sub>4</sub>-3-1-1; EM<sub>4</sub>-3-1-2; SCM<sub>3</sub>-4-3-1), F<sub>2</sub>, and F<sub>3</sub> entries from each generation family (FM 958 x EM<sub>4</sub>-3-1-1; FM 958 x EM<sub>4</sub>-3-1-2; FM 958 x SCM<sub>3</sub>-4-3-1) were planted in a RCBD with 4 replications in 2013 at Lubbock and Lamesa, TX on 8 May 2013. Plots were one row, 5.8 m x 1 m. Treatments included imazamox applied at 176 g a.i. ha<sup>-1</sup> (4x recommended label rate) plus COC at 1% v/v applied with a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup>. In 2013, treatments were applied when plants contained 8 to 12 true leaves on 8 July and 5 July at Lubbock and Lamesa, respectively. Data included individual plant tolerance ratings of tolerant, intermediate, or susceptible. In the parental, F<sub>2</sub>, and F<sub>3</sub> generations, 1473, 1421, and 1502 plants were rated, respectively.

### Statistical Analysis

To determine allelism ratios, individual plant tolerance ratings were subjected to a chi-square analysis using the equation:

$$\chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

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After the chi-square value was determined, p-values to determine goodness of fit for the ratios were calculated using the equation from Soper (2013):

$$P \text{ Value} = [ 0.5^{df/2} / \Gamma(df/2) ] \times (\chi^2)^{(df/2) - 1} \times e^{-\chi^2/2}$$

## Results and Discussion

Tolerance ratios from parental populations are presented in Table 13, and chi-square analysis of F<sub>2</sub> and F<sub>3</sub> populations are presented in Tables 14 and 15, respectively. Injury symptoms were discrete. Few (less than five per line within generation) were rated as intermediate. These could be considered herbicide application error, and were thus discarded leaving ratings in distinct groups of tolerant and susceptible. As represented by the sequence data in Chapter IV, the amino acid substitution mutation event at Ala122 conferring elevated IMI-tolerance was not consistent and appeared to be segregating within parental lines selected. At the initiation of population development for field trials, assumptions were made about the true breeding nature of the IMI-tolerance trait. However after evaluating field trials in 2012 and sequence analysis, it was clear that this assumption was false. Since parental seeds were subsequently bulked, it cannot be determined what ratio of the initial parental population, selected after the greenhouse screening event, were heterozygous for the mutation event. Without this information, ratios of tolerant and susceptible plant types cannot be used to infer gene action in parental populations. Selected tolerant parents were crossed with susceptible cultivar FM 958 to develop F<sub>1</sub> derived F<sub>2</sub> and F<sub>2</sub> derived F<sub>3</sub> generations; meanwhile bulked parental seeds were maintained and increased separately. This resulted in a

deviation from parental tolerant ratios and F<sub>2</sub> and F<sub>3</sub> tolerant ratios collected in field studies.

Some assumptions can be made by the ratios seen in F<sub>2</sub> and F<sub>3</sub> generations. F<sub>2</sub> population ratios (Table 14) were tested against an expected 3:1 ratio that would result after a cross between a homozygous dominant (selected parent) and homozygous recessive (FM 958) for the IMI tolerant mutation event. EM<sub>4</sub>-3-1-2 and SCM<sub>3</sub>-4-3-1 lines fit this ratio. F<sub>2</sub> derived F<sub>3</sub> populations were tested against a 5:3 ratio that would be expected to follow the 3:1 ratio after a generation of selfing (Table 15). All lines fit this expected ratio. The discrepancy between F<sub>2</sub> and F<sub>3</sub> generations in line EM<sub>4</sub>-3-1-1 could be due to rating or herbicide application error. Although tolerance appears to behave in a simple recessive way, it is speculated that tolerant plants are actually the simple dominant forms of the IMI-tolerant mutation. In screenings done by Bechere et al. (2009), intermediate ratings were obtained when applying imazamox at a rate of 88 g a.i. ha<sup>-1</sup> (2x labeled rate). Likewise during greenhouse screenings in this study, 88 g a.i. ha<sup>-1</sup> imazamox was applied leaving the likelihood that heterozygotes survived, and possibly selected. In field trials, imazamox at a rate of 176 g a.i. ha<sup>-1</sup> was used. This rate potentially overwhelmed the heterozygotes in field trials, causing them to be rated susceptible. With this premise, the ratios hold true to a dominant form of the IMI-tolerant mutation, which was also reported by Bechere et al. (2009).

Table 13. Tolerant and susceptible individual plant ratings at 28 DAA in parental populations in Lubbock and Lamesa, TX in 2013.<sup>a,b</sup>

	Tolerant	Susceptible
EM <sub>4</sub> -3-1-1	213	302
EM <sub>4</sub> -3-1-2	292	221
SCM <sub>3</sub> -4-3-1	199	221

<sup>a</sup>All plants received imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>DAA: Days after application.

Table 14. Chi-square analysis of individual plant ratings at 28 DAA in F<sub>2</sub> populations in Lubbock and Lamesa, TX in 2013 tested against an expected 3:1 ratio.<sup>a,b</sup>

	Observed		Expected		$\chi^2$	P value <sup>c</sup>
	T	S	T	S		
EM <sub>4</sub> -3-1-1	147	335	121	361	7.77	0.005
EM <sub>4</sub> -3-1-2	110	354	116	348	0.41	0.520
SCM <sub>3</sub> -4-3-1	105	348	113	340	0.80	0.371

<sup>a</sup>All plants received imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>DAA: Days after application.

<sup>c</sup>Chi-square P values greater than 0.05 indicate observed values are not significantly different than expected values.

Table 15. Chi-square analysis of individual plant ratings at 28DAA in F<sub>3</sub> populations in Lubbock and Lamesa, TX in 2013 tested against an expected 5:3 ratio.<sup>a,b</sup>

	Observed		Expected		$\chi^2$	P value <sup>c</sup>
	T	S	T	S		
EM <sub>4</sub> -3-1-1	219	320	202	337	2.29	0.130
EM <sub>4</sub> -3-1-2	162	314	178	298	2.30	0.130
SCM <sub>3</sub> -4-3-1	171	304	178	297	0.44	0.507

<sup>a</sup>All plants received imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>DAA: Days after application.

<sup>c</sup>Chi-square P values greater than 0.05 indicate observed values are not significantly different than expected values.

## **Gain from Selection**

### Materials and Methods

Greenhouse studies were initiated in the summer of 2010 in Lubbock, TX, to screen 31 previously derived IMI-tolerant mutant lines for potential parental selection. These lines had improved yield data from previous field studies. Fifty plants of each line were arranged in a Completely Randomized Design and imazamox was applied using a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup> at a rate of 88 g a.i. ha<sup>-1</sup> (2x recommended label rate) when plants contained 4 to 6 true leaves. Three lines (Table 1) showed elevated levels of visual imazamox tolerance (>95%) of which six siblings were selected of each line. Selected plants, along with a Texas High Plains standard cultivar FM 958 were planted in 6 L pots and grown to maturity under greenhouse conditions (30 C and watered as needed). Selected IMI tolerant plants (male) were crossed with a respective FM 958 plant (female) to derive F<sub>1</sub> progeny (Figure 3). F<sub>1</sub> progeny from each cross combination (2 plants per cross combination) were planted in 6 L pots and grown to maturity under greenhouse conditions to derive F<sub>2</sub> seeds. These were bulked by family and planted in a field nursery in Lubbock, TX on 7 June, 2012. Imazamox was applied to the nursery at 176 g a.i. ha<sup>-1</sup> (4x recommended label rate) plus COC at 1% v/v with a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup> when plants contained 4 to 8 true leaves. Individual plant injury ratings were made at 14 DAA on a scale of 1 (no injury) to 9 (plant death). Across families, 767 F<sub>2</sub> plants were evaluated. Thirty-six individual plants with an injury rating of 2 or less were selected, tagged and hand harvested. Seeds from selected plants were planted into a nursery containing F<sub>3</sub> progeny

rows in Lubbock, TX on 23 May, 2013 (Figure 4). Imazamox was applied to the nursery at 176 g a.i. ha<sup>-1</sup> (4x recommended label rate) plus COC at 1% v/v with a CO<sub>2</sub>-pressurized backpack sprayer delivering 140 L ha<sup>-1</sup> when plants contained 6 to 12 true leaves. Individual plant injury ratings were made at 14 DAA on a scale of 1 (no injury) to 9 (plant death). Across families, 2167 F<sub>3</sub> plants were evaluated.

### Statistical Analysis

In order to estimate gain from selection, a realized heritability estimate was calculated using the general formula  $h^2 = R/S$ , where  $R$  is the response to selection and  $S$  is the selection differential (Fehr 1991), which results in the equation:

$$h^2 = \frac{\bar{x}_{F3} - \bar{x}_{F2}}{\bar{x}_{s,F2} - \bar{x}_{F2}}$$

Where  $\bar{x}_{F2}$  = Base Population Mean

$\bar{x}_{s,F2}$  = Mean of Selected Parents

$\bar{x}_{F3}$  = Mean of offspring of Selected Parents

### Results and Discussion

Mean injury for individual plant ratings are presented in Table 16. A gain from selection estimate can be a good indicator of the progress realized by selection, but may not be valid for a true estimate of heritability (Falconer 1981). Environmental changes, genetic drift, or inbreeding depression could cause changes in the populations unrelated to selection (Fehr 1991). Data indicate a 13.6% improvement in herbicide tolerance



resulting from one generation of selection, lending to a low progress from selection. This may be due to the complicated nature of the ALS gene in *G. hirsutum*. Even if selection is occurring for the Ala122 mutation event on the ALS-LSU that confers an elevated level of IMI tolerance, the ALS gene family in *G. hirsutum* has been characterized to have six different ALS genes with varying functions as noted above. The complex nature of the ALS gene family in *G. hirsutum* could cause difficulty in selecting cotton varieties with a commercially acceptable level of IMI-tolerance.

Table 16. Injury means for F<sub>2</sub>, selected F<sub>2</sub>, and resulting F<sub>3</sub> populations for nurseries in Lubbock, TX in 2012 and 2013.<sup>a</sup>

	Mean Injury <sup>b</sup>
Base Population( $\bar{x}_{F_2}$ )	6.51
Selected Parents( $\bar{x}_{s,F_2}$ )	1.23
Offspring of Selected Parents( $\bar{x}_{F_3}$ )	5.79

<sup>a</sup>All plants received imazamox at 176 g a.i. ha<sup>-1</sup> plus crop oil concentrate at 1% v/v.

<sup>b</sup>Individual plants rated at 14 DAA for herbicide injury on a visual scale of 1 (no injury) to 9 (plant death).

## CHAPTER VI

### ON-FARM PARTIAL BUDGET ANALYSIS FOR NOVEL HERBICIDE TOLERANT TECHNOLOGY

#### **Introduction**

Glyphosate-resistant Palmer amaranth is one of the most economically damaging weeds in the United States (Beckie 2011). With the advent of glyphosate-resistant Palmer amaranth throughout the Cottonbelt (Culpepper et al. 2006; Norworthy et al. 2008; Steckel et al. 2008; Buerkle 2011), producers now face additional costs in crop protection to protect yield potential. In Texas, it has been shown that Palmer amaranth plant densities of 1 to 10 plants  $9.1 \text{ m}^{-2}$  can reduce cotton lint yields by 11 to 50%, respectively (Morgan et al. 2001). In addition to competition for resources needed for plant growth Palmer amaranth can impact yields through allelopathy (Bradow and Connick 1987), and in subsequent seasons as research has indicated that the weed can be a host to various nematode pests (Davis and Webster 2005).

Glyphosate-resistant Palmer amaranth control requires integration of mechanical and hand-cultivation (Ward et al. 2013), can cost approximately \$40.00  $\text{ha}^{-1}$  (Smith and Yates 2013) and may need to be done several times throughout the growing season. However, non-chemical controls have been shown to be inconsistent (Sosnoskie et al. 2012). To control glyphosate-resistant Palmer amaranth and protect cotton yield potential, adoption of cotton varieties with multiple stacked herbicide tolerant traits is needed along with proper resistance management practices (Ward et al. 2013). For GM

herbicide technology, producers in the Texas High Plains pay from \$1.53 to \$1.93 ha<sup>-1</sup> in technology fees, depending on plant densities under non-irrigated or irrigated production systems, respectively (Smith and Yates 2013). Because mutation-based HTC technology would reduce producer costs through the absence of technology fees, it could provide a cost effective option for cotton producers on the Texas High Plains.

## **Partial Budget Analysis**

### Materials and Methods

In order to determine hypothetical net returns from differing weed control scenarios compared to inputs of a possible IMI-tolerant cotton production system, a partial budget analysis was conducted using budget calculations from Smith and Yates (2013) (Tables 17, 18, and 19). Average cotton lint yields for glyphosate and insect tolerant cotton under irrigated conditions, conventional cotton under irrigated conditions, and conventional cotton under dry-land conditions, were derived from Smith and Yates (2013) and Dever et al. (2012). Cost of imazamox herbicide (Raptor®) was calculated to be \$48.26 ha<sup>-1</sup> (Ferrell and Sellers 2011). Price for cotton lint yield was set at \$1.67 kg<sup>-1</sup> and seed turnout was set at 327 kg per 227 kg of lint at a price of \$286.66 t<sup>-1</sup>. Dry-land production systems do not include costs associated with operating irrigation. Additional fixed inputs and on-farm operating costs for each production scenario can be found in budgets compiled by Smith and Yates (2013).

Partial budget analysis was done for a “worst case scenario” where a production field is heavily infested with glyphosate-resistant Palmer amaranth. Differing weed control

input scenarios were analyzed, and assumptions were made about certain input variables and yield reductions associated with each. Technology fees were assessed for glyphosate and insect tolerant cotton production system of \$1.93 ha<sup>-1</sup> and not for conventional systems. Plant densities were assumed to be 129,000 plants ha<sup>-1</sup> for irrigated systems, and 97,000 plants ha<sup>-1</sup> for dry-land systems. Yield reduction scenarios from potential populations of glyphosate-resistant Palmer amaranth are estimated based on results by Morgan et al. (2001). In each production system, input scenarios per growing season of no hoeing, hoeing once, hoeing twice, and hoeing once plus one imazamox application were assigned 45%, 25%, 11%, and 0% hypothetical yield reductions, respectively. Tractor fuel costs were adjusted to reflect usage ha<sup>-1</sup> in each production system and weed control input scenario. Net returns reflect the producer owing no rents or participating in cost sharing.

### Results and Discussion

Yield loss due to glyphosate-resistant Palmer amaranth populations that is assumed from studies by Morgan et al. (2001) results in large net losses in several systems and scenarios (Tables 17, 18, 19). In the conventional irrigated system, analysis shows net losses in all weed control input scenarios presented. In this system, the producer would have to rely on either having a population of Palmer amaranth that is not glyphosate-resistant or have no population present. Under low-input conditions in Table 19, having the option of applying imazamox put returns just above break-even, where other input scenarios result in a net loss. An IMI-tolerant cotton production system would be most

applicable and profitable in a high input system under irrigation with tolerance to multiple herbicide modes of action. Each increase in weed control input cost results in a growth in net returns as each is adding more protection to yield potential. Although the price of Raptor® herbicide is relatively expensive compared to glyphosate, including an imazamox application with a hoeing cultivation gave the hypothetical highest net returns.

Large assumptions were made about the link between hoeing scenarios and resulting Palmer amaranth densities, yield loss due to weed density competition, and other budget impacts involved with additional weed control inputs. These need to be addressed before official budget recommendations can be made. However, this exercise illustrates the enormous pressure glyphosate-resistant weeds can have on producer net profit. Multiple herbicidal modes of actions, plus non herbicidal controls, will be needed to combat glyphosate-resistant weeds and maintain yield potential and profits. Adding mutation-based IMI-tolerance to a cotton production system in conjunction with glyphosate- and insect-tolerant systems would provide producers with an additional weed control tool without adding technology fee costs to their bottom line, and thus works to maximize producer net return.

Table 17. Partial budget analysis for differing weed control input variables compared to novel herbicide tolerant technology in Texas High Plains glyphosate and insect tolerant cotton production under irrigated conditions.<sup>a</sup>

Weed Control Inputs	Price ha <sup>-1</sup> (\$)			
	No Hoe (45% Yield Reduction)	1 Hoe (25% Yield Reduction)	2 Hoes (11% Yield Reduction)	1 Hoe + Raptor® (0% Yield Reduction)
Pre-plant Herbicide	29.65	29.65	29.65	29.65
Post Herbicide	39.54	39.54	39.54	39.54
Raptor® Herbicide <sup>b</sup>	n/a	n/a	n/a	48.26
Hoeing	n/a	39.54	79.07	39.54
Fuel - Tractor	31.92	31.92	31.92	42.57
Total Cost	101.11	140.65	180.19	199.56
Predicted Yield (kg ha <sup>-1</sup> ) <sup>c</sup>	771.25	1051.70	1248.02	1402.27
Net Return <sup>d</sup>	(368.08)	63.08	352.68	592.31

<sup>a</sup>Fixed input and on-farm operating costs found in production budgets by Smith and Yates (2013).

<sup>b</sup>Price reflects application of Raptor® at 44 g a.i. ha<sup>-1</sup> (1x rate).

<sup>c</sup>Yield reduction assumed from scenarios indicated in Morgan et al. (2001).

<sup>d</sup>Net Return includes \$1.67 kg<sup>-1</sup> cotton lint seed turnout at 327 kg per 227 kg cotton lint at \$286.66 t<sup>-1</sup>.

Table 18. Partial budget analysis for weed control input variables compared to novel herbicide tolerant technology in Texas High Plains conventional cotton production under irrigated conditions.<sup>a</sup>

Weed Control Inputs	Price ha <sup>-1</sup> (\$)			
	No Hoe (45% Yield Reduction)	1 Hoe (25% Yield Reduction)	2 Hoes (11% Yield Reduction)	1 Hoe + Raptor® (0% Yield Reduction)
Pre-plant Herbicide	29.65	29.65	29.65	29.65
Post Herbicide	12.35	12.35	12.35	12.35
Raptor® Herbicide <sup>b</sup>	n/a	n/a	n/a	48.26
Hoeing	n/a	39.54	79.07	39.54
Fuel - Tractor	14.08	14.08	14.08	21.12
Total Cost	56.08	95.62	135.15	150.92
Predicted Yield (kg ha <sup>-1</sup> ) <sup>c</sup>	416.48	567.98	673.93	757.23
Net Return <sup>d</sup>	(771.81)	(557.72)	(420.06)	(298.86)

<sup>a</sup>Fixed input and on-farm operating costs found in production budgets by Smith and Yates (2013).

<sup>b</sup>Price reflects application of Raptor® at 44 g a.i. ha<sup>-1</sup> (1x rate).

<sup>c</sup>Yield reduction assumed from scenarios indicated in Morgan et al. (2001).

<sup>d</sup>Net Return includes \$1.67 kg<sup>-1</sup> cotton lint seed turnout at 327 kg per 227 kg cotton lint at \$286.66 t<sup>-1</sup>.

Table 19. Partial budget analysis for weed control input variables compared to novel herbicide tolerant technology in Texas High Plains conventional cotton production under dry-land conditions.<sup>a</sup>

Weed Control Inputs	Price ha <sup>-1</sup> (\$)			
	No Hoe (45% Yield Reduction)	1 Hoe (25% Yield Reduction)	2 Hoes (11% Yield Reduction)	1 Hoe + Raptor® (0% Yield Reduction)
Pre-plant Herbicide	29.65	29.65	29.65	29.65
Post Herbicide	12.35	12.35	12.35	12.35
Raptor® Herbicide <sup>b</sup>	n/a	n/a	n/a	48.26
Hoeing	n/a	39.54	79.07	39.54
Fuel - Tractor	14.08	14.08	14.08	21.12
Total Cost	56.08	95.62	135.15	150.92
Predicted Yield (kg ha <sup>-1</sup> ) <sup>c</sup>	215.95	294.48	349.45	392.64
Net Return <sup>d</sup>	(177.81)	(87.67)	(36.79)	16.23

<sup>a</sup>Fixed input and on-farm operating costs found in production budgets by Smith and Yates (2013).

<sup>b</sup>Price reflects application of Raptor® at 44 g a.i. ha<sup>-1</sup> (1x rate).

<sup>c</sup>Yield reduction assumed from scenarios indicated in Morgan et al. (2001).

<sup>d</sup>Net Return includes \$1.67 kg<sup>-1</sup> cotton lint seed turnout at 327 kg per 227 kg cotton lint at \$286.66 t<sup>-1</sup>.



## CHAPTER VII

### CONCLUSIONS

Germplasm exhibiting tolerance to the imidazolinone class of herbicides has been identified (Bechere et al. 2009; 2010). However, the physiological basis, genetic behavior, and potential for herbicide tolerance improvement were not fully understood.

Greenhouse studies were initiated in the summer of 2010 in Lubbock, TX, to screen 31 previously derived IMI tolerant mutant lines for potential parental selection. Three lines (EM<sub>4</sub>-3-1-1, EM<sub>4</sub>-3-1-2, and SCM<sub>3</sub>-4-3-1) showed high levels of imazamox visual tolerance (>95%) of which six siblings were selected of each line to be used as parental sources for field studies. Yield, lint turnout, fiber strength, length, and micronaire of selected parents were analyzed to determine if EMS treatment caused adverse effects. Yield data indicate that all EMS treated lines had yield that was equal to or greater than their respective non-EMS treated cultivar. No significant differences were indicated between lines evaluated for lint turnout, fiber strength, fiber length, or micronaire properties. Thus it can be concluded that EMS treatment had no adverse effects on agronomic properties.

In 2012, IMI injury in selected parental populations was transient throughout the growing season, and plants appeared to recover from injury sustained. However elevated levels of injury were observed. Although data indicate that plants show the ability to recover from injury sustained from imazamox application, this level of injury

seen and trend of yield reductions raises questions about acceptable levels of IMI-tolerance being conferred, and would likely not be commercially viable.

Gene sequencing was performed to determine any possible mutation event on the ALS-LSU that would confer IMI tolerance. Consensus ALS-LSU sequence alignment in evaluated lines indicate a single base pair G to A point mutation at nucleotide 337 of reference *G. hirsutum* ALS amino acid sequence. This base pair change results in an amino-acid substitution of Ala122 to Thr122 in lines containing the G to A point mutation. This is likely the candidate mutation leading to tolerance of IMI herbicides in selected lines. However, sequence data indicate that this mutation is not consistent within all selected IMI-tolerant lines. This leads to conjecture that selected parental lines were not true breeding for the IMI-tolerant mutation and are still segregating for this trait. There also appears to be a sequence deletion that results in a truncated form of the protein. The structure of the ALS-LSU protein folds in a way that places the Ala122 to Thr122 substitution in a pocket of a proposed key herbicide-binding site, thus this truncated region could inhibit binding of the IMI herbicide and therefore confer a higher level of IMI-tolerance in these parental lines compared to those without the truncated protein form or the Ala122 point mutation. Further ALS gene sequencing is needed to confirm polymorphisms and limit the possibility of PCR error.

Broad sense heritability estimates of zero in all generations indicated that there was no measurable genetic variance among these lines. Parent-offspring regression analysis using parents and F<sub>2</sub> progeny indicate a moderate  $h^2$  value of 0.53. These results could be linked to the sequence data in that the parental lines selected for these studies were

inconsistent for the IMI mutation event, and therefore this trait was inconsistently transmitted to the F<sub>2</sub> generation. However, the parent-offspring h<sup>2</sup> estimate using F<sub>2</sub> plants and F<sub>3</sub> progeny was 0.84, demonstrating a strong relationship between generations and resemblance of plant tolerance.

Data from chi-square analysis allow some assumptions to be made about the ratios seen in F<sub>2</sub> and F<sub>3</sub> generations. F<sub>2</sub> population ratios were tested against an expected 3:1 ratio that would result after a cross between a homozygous dominant (selected parent) and homozygous recessive (FM 958) for the IMI-tolerant mutation event. EM<sub>4</sub>-3-1-2 and SCM<sub>3</sub>-4-3-1 lines fit this ratio. F<sub>2</sub> derived F<sub>3</sub> populations were tested against and fit a 5:3 ratio that would be expected to follow the 3:1 ratio after a generation of selfing. Although tolerance appears to behave in a simple recessive way, it is speculated that tolerant plants are actually the simple dominant forms of the IMI-tolerant mutation. A high imazamox rate potentially overwhelmed the heterozygotes in field trials, causing them to be rated susceptible. With this premise, the ratios hold true to a simple dominant form of the IMI-tolerant mutation.

Gain from selection data indicate a 13.6% improvement in herbicide tolerance resulting from one generation of selection, lending to low progress from selection. This may be due to the complicated nature of the ALS gene in cotton, and the inconsistency of the Ala122 to Thr122 IMI-tolerant mutation event in evaluated lines. The allotetraploid nature of *G. hirsutum* combined with the 6 copy number of the gene could make it difficult to select cotton varieties with genetic variances on all housekeeping forms of the ALS gene that would confer a commercially acceptable level of IMI-

tolerance.

A partial budget analysis illustrating a worst case scenario of Palmer amaranth infestation demonstrates the enormous pressure glyphosate-resistant weeds can have on producer net profits. Multiple herbicidal modes of actions plus non-herbicidal controls will be needed to combat glyphosate-resistant weeds and maintain yield potential and profits. Adding mutation-based IMI-tolerance to a cotton production system in conjunction with glyphosate tolerant systems would provide producers with an additional weed control tool without adding technology fee costs to their bottom line, and thus works to maximize producer net return.

These studies have demonstrated that non-transgenic breeding methods can confer and improve IMI herbicide tolerance in cotton, although levels of IMI herbicide injury deemed not commercially acceptable were present in evaluated populations and was slow to improve from selection. Future research could take steps to potentially deploy cotton varieties conferring acceptable levels of IMI-tolerance. Lines evaluated in this study show an IMI-tolerant mutation on one known ALS gene copy. Research has indicated that the 6 copy nature of the ALS gene family in *G. hirsutum* allows IMI injury to persist as the other copies are still functional. During germplasm development by Bechere et al. (2009), plants were rated tolerant based on their ability to survive imazamox (2x labeled rate) with minimum damage as compared to the non-mutant parents which exhibited severe damage. This suggests selected tolerant plants, which were later identified to contain the Ala122 IMI-tolerant mutation on one known ALS gene, still displayed injury. Any visual injury would not be commercially viable. Future

IMI-tolerant cotton developed through mutagenesis would have to contain multiple IMI-tolerant mutation events on different ALS gene copies. Marker development and genotyping for these events needs to be performed in early generations to confirm presence of mutation and accuracy of selection.

To achieve commercial IMI-tolerance in cotton, genetic modification could be deployed. In wheat, the compound chlorsulfuron is rendered non-toxic through the cytochrome p-450 metabolic pathway (Sweetser et al. 1982). Soybean is known to be naturally tolerant to IMI herbicides through its ability to rapidly metabolize and detoxify the compound (Teclé et al. 1993). If the cytochrome p-450 metabolic pathway genes that regulate tolerance to ALS herbicides in these crops could be isolated, introducing them into *G. hirsutum* via recombinant DNA and gene introgression via backcross breeding has the potential to create cotton germplasm with commercial levels of tolerance.

This trait could be a valuable tool to Texas cotton producers under severe pressure from glyphosate-resistant weeds. However, the complexity of cotton's ALS gene may inhibit the development of IMI herbicide tolerance in cotton through a single IMI-tolerant mutation. Alternate breeding steps such as screening for mutation events on multiple ALS gene copies, early generation marker assisted selection, or introgression of a metabolic regulator gene might be required to produce commercially viable IMI-tolerance in cotton.

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